Statins Inhibit Cytomegalovirus Replication by Interfering with the Isoprenoid Arm of the Mevalonate Pathway

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"Tout début contient une promesse et un défi.

Pour moi ce ne fut pas différent."

Joseph Meyerowitz

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Summary

Primary infection of human cytomegalovirus (HCMV) is rarely recognized in the immune competent host, and seroprevalence reaches 80% in the older population. Life-long persistence is the hallmark of HCMV. In immunocompromised patients such as in transplant recipients and HIV-infected patients, infections are a major concern and are associated with severe morbidity and mortality. Both reactivation and primary infection can occur. In the absence of vaccines against HCMV, the management and prevention of infection relies on the three systemic drugs approved for CMV treatment, ganciclovir, foscarnet and cidofovir. Considering the extended treatment period required for the treatment in immunocompromised patients, several drawbacks are limiting the use of these drugs: severe acute and long-term toxicities, and a shared viral target with the risk of emergence of drug cross-resistance. There is an urgent need for new therapeutic approaches with a good safety profile and an alternative mechanism of action that would ideally interfere earlier in the HCMV replication cycle.

Statins are a well-tolerated and extensively studied group of cholesterol-lowering drugs, exhibiting strong anti-inflammatory and immune-modulatory activities. Antiviral effects of statins have been reported on different viruses alone or in combination with selective antiviral inhibitors and have been associated with cholesterol-lowering or cholesterol-independent mechanisms. So far, despite a large number of statins currently used in clinics, little is known about the potential of statins against HCMV. The goal of this study was to investigate the in vitro anti-CMV activity of four statins (atorva-, fluva-, prava- and simvastatin) in human aortic endothelial cells (HAEC) and fibroblasts.

Our findings demonstrate that all statins dose-dependently reduce HCMV titers in both cell types. Atorva-, fluva- and simvastatin showed comparable EC50 and EC90, within a low micromolar range in HAEC, whereas pravastatin exhibited only limited effects. The same hierarchy was observed in fibroblasts, although all statins exhibited slightly less anti-CMV potency. The reduction of HCMV titers did not result from an inhibition of HCMV entry or an activation of the type I IFN response, but from the alteration of the expression of several viral antigens. Interestingly, statins treatment not only blocked the accumulation of immediate early antigens, but also interferes independently with early and late antigen expression.

Despite our attempts, the key mediator of the anti-CMV activity of statins was not identified. However, metabolite rescue experiments suggested an involvement of the non-sterol isoprenoid arm of the mevalonate pathway as the mode-of-action. Anti-CMV activity of all statins was almost completely abrogated by mevalonate and partially reversed by geranylgeranyl-pyrophosphate, an isoprenoid intermediate, whereas cholesterol failed to counteract the effects of statins. In an attempt to evaluate potential clinical benefits, we demonstrate an additive anti-CMV activity of statins at therapeutically relevant concentrations. While, the antiviral activity of statins was comparable to ganciclovir, statins enhanced the anti-CMV activity of low doses of ganciclovir suggesting a potential benefit for combination therapy. Finally, statins anti-CMV activity was retained in a ganciclovir-resistant HCMV strain.

In conclusions, these findings provide new insight into the beneficial effects of statins, adding antiviral activity against HCMV to their list of pleïotropic properties. Although the *in vivo* anti-CMV activity of statins might be limited to the lipophilic compounds, this supports further clinical investigation of the beneficial use of statins, as part as a cholesterol-lowering or anti-inflammatory therapy, against HCMV infection. Special attention should be taken to evaluate statins in combined therapy for the management of active HCMV disease and against cross-resistant HCMV strain. Finally, we believe this work may reveal potential targets for alternative anti-viral therapies.

Zusammenfassung

Die Neuinfektion mit humanen Zytomegalieviren (HZMV) erfolgt häufig ohne klinische Symptome, die Seroprävalenz steigt in der älteren Population bis gegen 80%. Die lebenslange Persistenz ist eines der entscheidenden Merkmale aller Herpesviren. Wird es notwendig, das Immunsystem medikamentös zu unterdrücken, wie nach Transplantation, oder kommt es im Laufe einer HIV Infektion zu einer Immunschwäche, kann eine Reaktivierung oder eine Neuinfektion fatale Folgen haben. Eine baldige Impfung ist klinisch nicht zu erwarten, so dass die Prävention und Behandlung von ZMV primär medikamentös durchgeführt wird. Drei Wirkstoffe sind dafür zugelassen: Ganciclovir, Foscarnet und Cidofovir. Ist eine längere Behandlung notwendig, sind relevante Nebenwirkungen kurz- und langfristiger Art häufig, ebenso kann es zu Resistenzentwicklung kommen. Neue Therapieansätze sind nötig, die alternative antivirale Mechanismen benützen und ein günstiges Toxizitätsprofil aufweisen.

Statine gehören zu eine gut tolerierten und in der klinischen Anwendung mehrfach erprobten Stoffklasse. Neben ihren lipidsenkenden Eigenschaften sind anti-entzündliche und immune-modulatorische Effekte beschrieben. Eine antivirale Potenz wurde gegen verschiedene Viren nachgewiesen, entweder bei alleiniger Gabe oder in Kombination mit weiteren antiviralen Substanzen. Die Mechanismen beruhen teils auf den lipidsenkenden Eigenschaften der Statine, teils aber auch auf den oben erwähnten Zusatzeffekten.

Die antiviralen Eigenschaften von Statinen gegen HZMV sind bis jetzt nur wenig erforscht. Das Ziel dieser Arbeit war es, die antivirale Aktivität gegen HZMV von vier in der Klinik gebräuchlichen Statinen nachzuweisen und die Mechanismen aufzudecken. Wir benützten dafür Atorva-, Fluva-, Prava- und Simvastatin, und entwickelten ein Endothelzellmodell basierend auf humanen Endothelzellen, die aus der Hauptschlagader stammen. Als Vergleich wurden Fibroblasten (Stützzellen des Bindegewebes) herangezogen.

Unsere Resultate zeigten eine Dosis-abhängige Reduktion der HZMV Replikation in beiden Zelltypen. In Endothelzellen wiesen Atorva-, Fluva- und Simvastatin eine vergleichbare Hemmung auf, während der antivirale Effekt von Pravastatin marginal war. Ein vergleichbares Resultat wurde auch in Fibroblasten gezeigt. Die Expression

viraler Antigene hingegen war deutlich inhibiert, und zwar war nicht nur die Produktion früher viraler Proteine, sondern auch diejenige späterer im Replikationszyklus auftretender Proteine. Die Wirkung beruht nicht auf einem verminderten Eindringen von HZMV in die Zielzellen, auch ist der antivirale Effekt nicht auf eine Aktivierung einer zellspezifischen antiviralen Antwort (z.b. Interferon) zurückzuführen.

Es war uns nicht möglich, einen einzelnen Schlüsselmechanismus für diesen Effekt zu identifizieren. Statine hemmen verschiedene für den Zellhaushalt wichtige Vorgänge. Durch gezielte Zugabe verschiedener Produkte konnte der sog. Isoprenoid Arm des Mevalonat-Stoffwechselpfades als derjenige beschrieben werden, der für den antiviralen Effekt verantwortlich ist. So war die hemmende Wirkung bei allen Statinen praktisch aufgehoben, wenn Mevalonate kompensatorisch dazugegeben wurde, und mit Geranylgeranyl-pyrophosphat, einer weiteren Substanz dieses Pfades, konnte der Effekt der Statine teilweise antagonisiert werden. Cholesterin, auf der anderen Seite, beeinträchtigte die Wirkung überhaupt nicht.

Die additive oder mögliche synergistische Wirkung wurde in Kombinationsversuchen mit Ganciclovir getestet, in der Tat kam es zu einer verstärkten Wirkung bei der gemeinsamen Gabe. Statine hatten auch bei einem gegen Ganciclovir resistenten Stamm eine antivirale Wirkung.

Zusammenfassend erhellen diese Resultate die antivirale Potenz der Statine und kreisen einen möglichen Mechanismus ein. Die antivirale Potenz muss zu den vielfältigen Eigenschaften von Statinen dazugezählt werden. Die weitere Erforschung der Wirkung in klinischen Versuchen scheint gerechtfertigt, insbesondere bei gegenüber den herkömmlichen Therapien resistenten HZMV Stämmen. Neue Erkenntnisse der antiviralen Mechanismen können neue potentielle antivirale Ziele aufzeigen.

Introduction

I. The Human Cytomegalovirus

1. Historical perspective and classification

Clinical manifestations of cytomegalovirus infection were first noticed during the late 19th century. The observation of typical cytomegalic cells with an eccentrically nuclei containing intranuclear occlusions with an halo as "protozoan like" cells was reported in the kidney sections of a luetic stillborn by the german physician Ribbert [1] and described based on similar observation by Jesionek and Kiolemenoglou in 1904 (Figure 1). A viral etiology was later proposed for cytomegalic inclusion disease after the observation of such cells in patients with herpes zoster and herpes genitalis [2]. The high frequency of cytomegalovirus infection (present in 12% of infants dying from a variety of causes) was further reported, as well as the variety of infected organs [3].

The identification of the causal agents had to wait for the improvement of cell cultures techniques till 1956/1957, when three groups independently identified a virus only growing in human but not mouse cells [4], from an infant suspected to have congenital toxoplasmosis [5] and from the salivary gland and the kidney of two different patients [6]. This virus was named "Cytomegalovirus" in 1960.

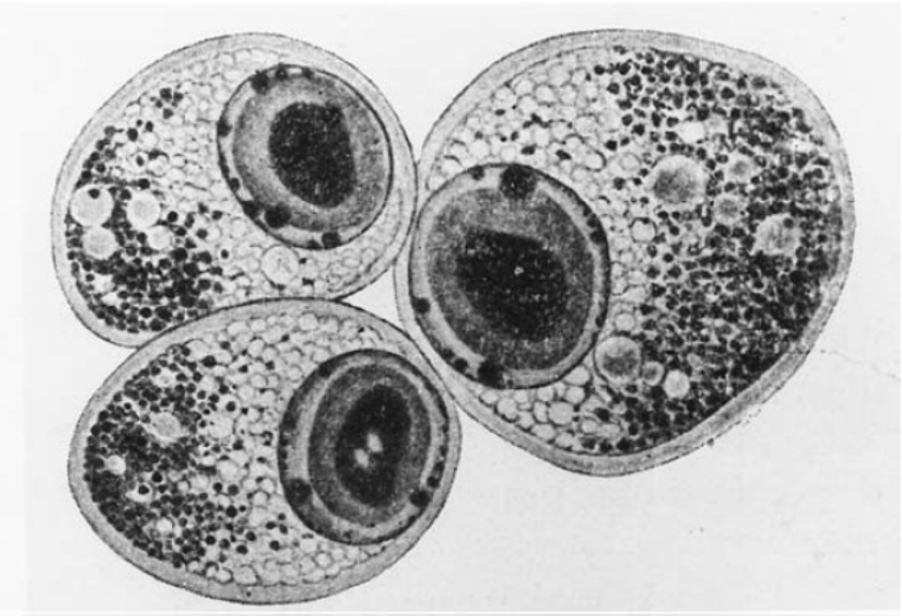

Figure 1: Intranuclear inclusion body as seen in the year 1904.

Reproduced from Jesionek and Kiolemenoglou,Muenchner Med Wochenschr,1904 [7]

The human cytomegalovirus (HCMV) belongs to the *Herpesviridae* family regrouping more than 200 DNA viruses causing disease in animals, including humans [8]. All herpesviruses share common structures and features:

- a large double-stranded linear DNA genomes encoding up to 200 genes;
- an icosahedral proteic capsid enclosed in a matrix of viral phosphoproteins and mRNAs called the tegument;
- a lipid bilayer envelope containing glycoproteins complexes;
- the ability to undergo lifelong latent infection in their host;

Herpesviruses are subdivided into three subfamilies (*α-*, *β-* and *γ- herpesvirinae*), based on biological criteria such as host range, growths kinetics, cell tropism or site of latency. HCMV, also known as human herpes virus-5 (HHV-5), belongs to the *β-herpesvirinae*, together with the HHV-6A and 6B and HHV-7. They are characterized by large genomes, a restricted host range and a slow replication cycle of up to 72 hpi in culture and are believed to establish latency in lymphoid cells.

The *α-herpesvirinae* comprise three human viruses (herpes simplex viruses 1/2, HHV-1/2; varicella zoster virus, HHV-3) that are characterized by i) smaller genomes as compared to *β-herpesvirinae*, ii) a fast replication cycle with release of progeny virus starting already after 8 hours post infection (hpi), iii) a latency established in neuron. The *γ-herpesvirinae* group viruses with a more diverse replication rate and host range, but which do not replicate *in vitro* in lymphoblastoid cells. Two major human pathogens, the Epstein-Barr virus (EBV, HHV-4) and the Kaposi's sarcoma-associated herpesvirus (KSHV, HHV-8) belong to this subfamily.

2. HCMV structure and replication cycle

Contrary to the restricted host range attributed to herpesviruses, a broad spectrum of cell types is permissive for HCMV *in vivo* and *in vitro*. With the exception of cells of lymphoid origin most cells types are invaded by viral particles and express at least immediate early (IE) antigens. However, full productive infection preferentially occurs in terminally differentiated cells including endothelial cells (EC) and fibroblasts, and results in the release of infectious progenies [9]. The HCMV infectious particles are an assembly of three major layers: a nucleocapsid containing the double stranded DNA genome, an amourphous proteinaceous layer encompassing the nucleocapsid known as

the tegument, a trilaminar lipid envelope of an overall size of approximately 230 nm (Figure 2).

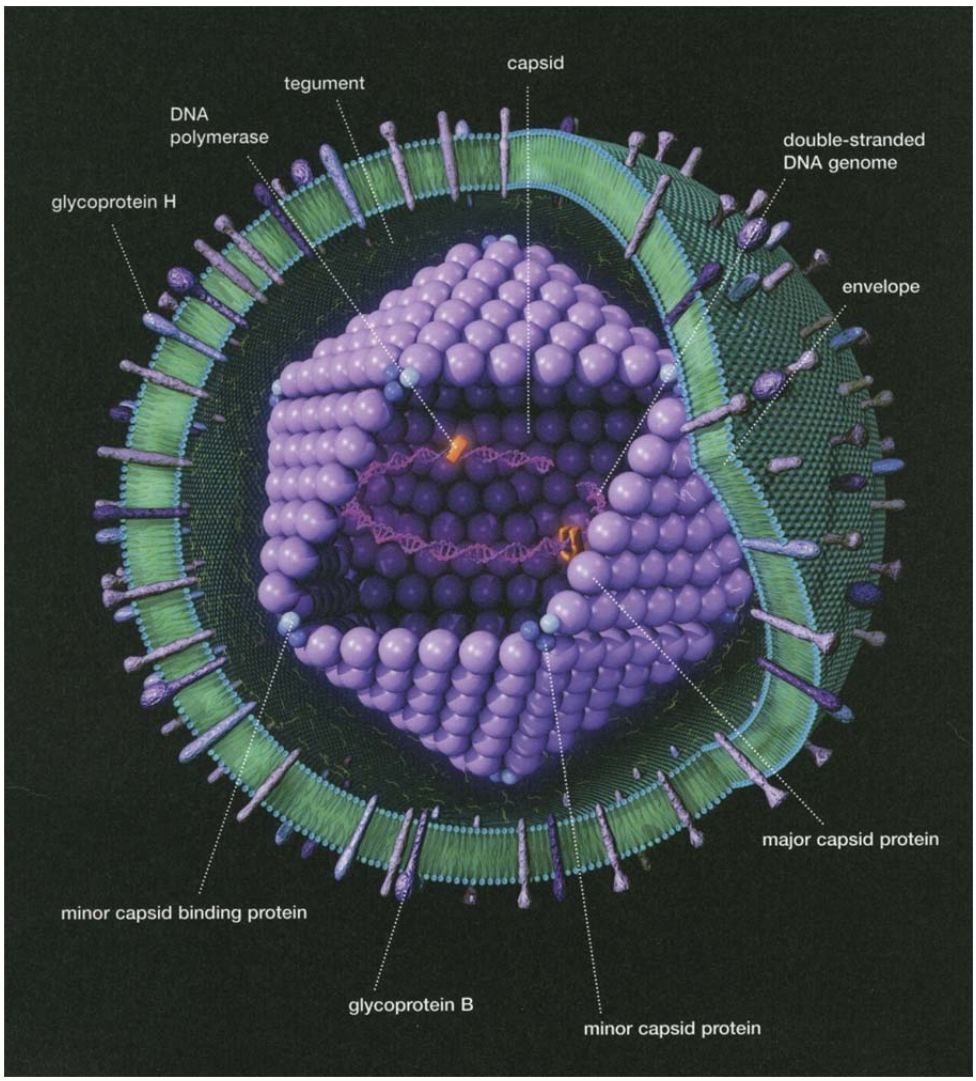

Figure 2: The architecture of HCMV particles

Reproduced from Human Cytomegalovirus; International Medical Press, 2007[10].

2.1 HCMV entry into host cell

HCMV entry into human cells is a sequential process requiring the interaction between multiple cellular and viral components to allow the adsorption and the penetration of the viral capsid into the cytoplasm. The many HCMV-permissive cell types suggest the use of receptor(s) common to most cells.

The initial interaction occurs with HCMV membrane-anchored glycoprotein complexes (gM/gN and gB) binding to the cell surface heparin sulphate proteoglycans, allowing the transient tether of the virions. Stable docking of the viral particles and entry through cellular trans-membrane protein receptors is then mediated by the glycoproteins gB and

gH/gL/gO complexes [11]. Different entry pathways have been identified depending on the cell type, via endocytosis in a pH-dependent manner (for EC) or by a pH-insensitive membrane fusion (fibroblasts) [12, 13]. Both entry pathways involve common cellular receptors; epidermal growth factor receptor (EGFR), αv-β3 or β1 integrins act as HCMV receptor or co-receptors [14-16]. The fusion of the envelope, either with the endocytic or cytoplasmic membrane, will allow the release of the nucleocapsid into the cytoplasm (Figure 3). The binding and fusion of the viral particles with the host cell membrane triggers significant induction of cellular pathway such as TLR-2 or the activation of cellular transcription factors essential for the initiation of HCMV replication.

2.2 Initiation of the viral gene expression and DNA replication

Teguments proteins are fully formed and active proteins mediating all events from release of the viral nucleocapsid within the cytoplasm to the initiation of the viral gene expression. The nucleocapsid is believed to translocate through the cytoplasm along the microtubules network, using the intracellular transport machinery [17]. The tegument phosphoprotein pp150 that is tightly associated with the capsid during this transit, play a crucial role within this process and is considered essential for lytic replication *in vitro* [18]. The complex formed by two others tegument proteins pUL47/48 together with the major capsid protein, seems also to be involved in the transport of the capsid and/or the injection of the viral DNA into the nucleus through the nuclear pore complex.

HCMV contains a large (236 kb) and complex genome, encoding over 200 predicted open reading frames (ORF), thus having the largest coding capacity among herpesviruses [19]. This linear double stranded DNA genome is organized in two domains, the unique long and short segment (U_L and U_S), each one flanked by a repeat sequence. HCMV genes nomenclature refers to the localization in the genome and is composed of the domain (UL or US) and of the serial number of the ORF starting from the 5' end. The genes products are referred by a "p" in front of the gene name (e.g. pUL44) except in the case of phosphoprotein, where the number correspond to their molecular weight (e.g. pp65). The genome also contains various transcriptional control regions that direct viral gene expression, DNA replication, packaging and transcription. The replication, typical of herpesviruses, is a complex temporally regulated cascade of events, with three families of genes being subsequently expressed: the immediate early (IE) genes within the first hours after the beginning of the infection, followed by the early (E) genes and the late (L) genes at last.

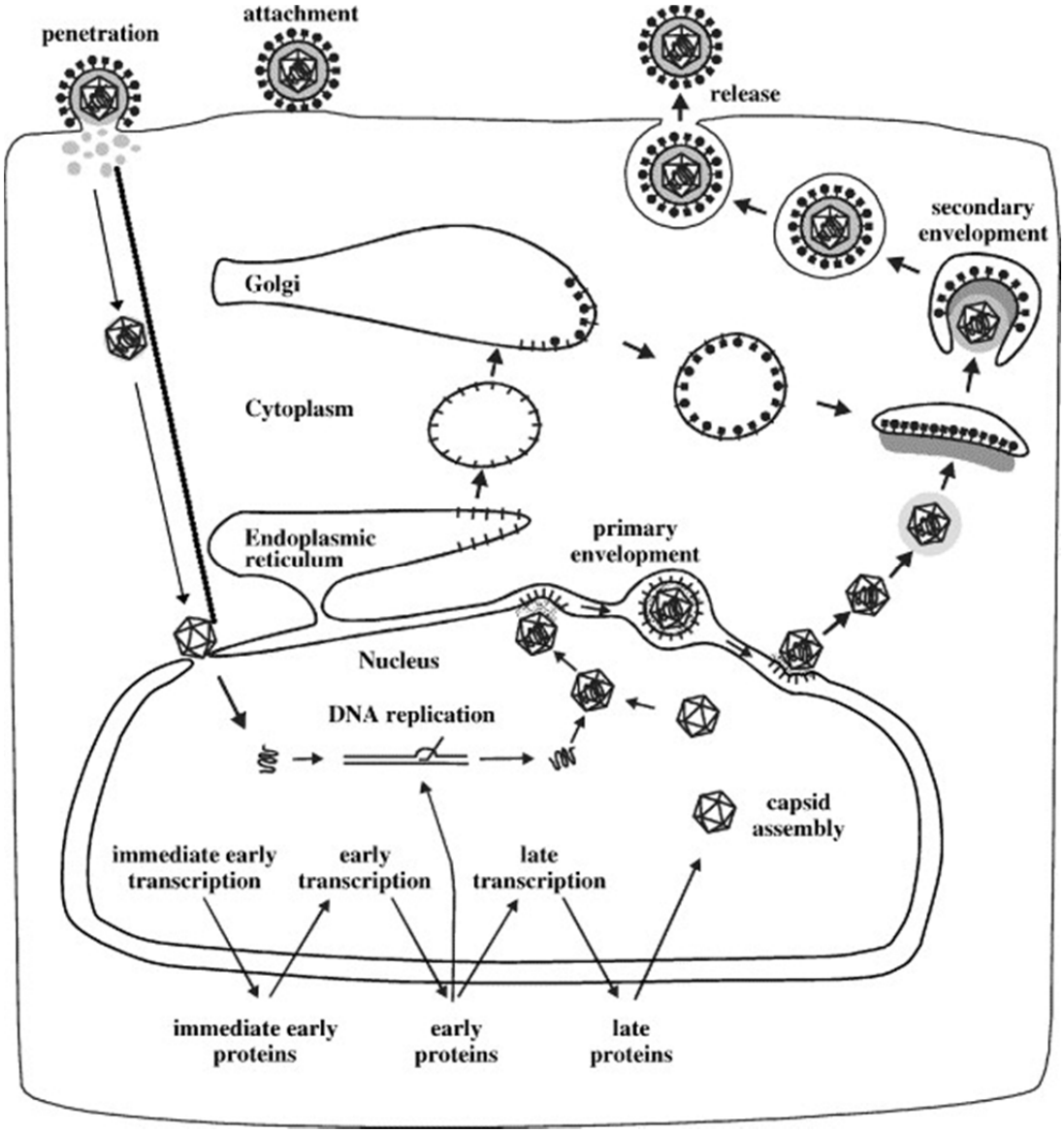

Figure 3: Complete replication cycle of the human cytomegalovirus

Reproduced from T.C. Mettenleiter, 2004 [20]

The transcription of the IE genes locus begin shortly after release of the viral DNA into the nucleus, under the control of the major immediate early enhancer-promoter (MIEP), without the synthesis of *de novo* viral protein [21]. This region is transactivated by the tegument proteins pUL69 and pUL82 that accumulates into the nucleus but contains also multiple binding sites for cellular transcription factors such as NF-κB. IE1 and IE2 are the two main proteins expressed after the differential splicing of the major IE transcript. They act synergistically to activate the expression of the viral E proteins, and autoregulate the MIEP in an antagonistic manner. IE1 (IE72, pUL123), the first of these two nuclear phosphoproteins to be expressed, accumulates within the first 6 hpi and appears to have multiple roles. IE1 disperses the nuclear domain 10 to facilitate the CMV

genome transcription and activate the MIEP promoter. IE1 is also a major antagonist of the cellular innate immune response, blocking the signal transduction of the type 1 interferon (IFN) response [22]. Contrary to IE1, which is not essential for productive replication but appears to be necessary for replicative success at low multiplicity of infection (MOI), IE2 (IE86, pUL122) is crucial for the lytic replicative cycle. IE2 negatively autoregulates its own expression through the MIEP and acts as the main transactivator of the viral E/L promoter. In addition, IE2 interacts with several cellular promoters to induce the cell cycle progression from G0/G1 to G1/S phase and to block the G2/M transition in order to create a cellular environment favorable for viral replication [23].

The onset of the viral DNA synthesis befalls at around 24 hpi, after the expression of the E gene products requires for HCMV core replication machinery. Eleven proteins have been identified as essential for the viral DNA replication (Figure 3). Once released in the nucleus, the viral DNA circularizes thanks to an unpaired nucleotide at both the 3' and 5' ends and will be used as a template for the production of a concatameric DNA, a mechanism shared with other herpesviruses [24]. The unique origin of replication of HCMV genome, ori*Lyt,* directs the initiation of the DNA replication [25], where the helicase-primase complex (pUL70, pUL105 and pUL102) unwinds the ds-DNA genome at the 5' end to allow the elongation of the viral genome by the viral DNA polymerase pUL54, using the free 3'-end as primer. pUL54 activity depends upon the association with pUL44 (pp52, ICP36), the DNA polymerase processivity factor which prevents the dissociation of the DNA polymerase from the DNA template and the single stranded DNA binding protein pUL57, which prevents in the meanwhile the re-annealing of the DNA strands. Further proteins play a role in DNA replication: IRS1/TRS1 acts as transactivator, pUL112/113 ensures the recruitment of the replication proteins to the nuclear sites of DNA synthesis, pUL36 and pUL37 prevents the cell apoptosis in responses to HCMV infection to ensure the complete replication cycle.

Prior to encapsidation, the cleavage of the concatameric DNA into unit genome lengths is performed by the terminase complex (pUL56 and pUL89).

2.3 Morphogenesis of the virions

The structural components of the viral particles, e.g. capsomeres, tegument and glycoproteins, are encoded by the L family of genes, whose expression occurs after the onset of the viral DNA replication.

The capsid assembly and maturation process is tightly coordinated by the assembly protein precursor (pAP, pUL80.5) and the maturational protease precursor (pPR, pUL80a), later eliminated from the matured particles. In the cytoplasm, pAP interacts with the major capsid protein (MCP, pUL86) to form the protocapsomers. The other integral subunit of the capsid shell is formed by two copies of the minor capsid protein (mCP, pUL85) interacting with the mCP-binding protein (mCBP, pUL46) to constitute a triplex. After translocation into the nucleus, the two types of oligomers coalesce together with the portal-protein complex (pUL104), a ring shaped structure that forms a channel for the viral DNA to enter and leave the capsid [26]. The smallest capsid protein (SCP, pUL48/49), an essential protein for the virion infectivity, binds to the external surface of the procapsid [27, 28]. During the maturation process, the internal scaffolding proteins (pPR and pAP) are eliminated and the newly replicated DNA is incorporated into the capsid. The mature viral capsid is an icosahedral scaffold structure, similar in size (≈110 nm) and shape to HSV, organized in 162 capsomeres and 320 triplexes located between the capsomeres (Figure 2).

The nucleocapsid exits the nucleus via an envelopment/ de-envelopment process through the nuclear cisterna (Figure 3). The process of assembly of the tegument is poorly understood and it is not clear whether it only starts after the egress of the nucleus or if some proteins already accumulate around the nucleocapsid while still in the nucleus. The final envelopment of the fully tegumented capsids occurs by budding into the trans-golgi network-derived vesicles where viral glycoproteins accumulate. The vesicle containing the enveloped virion is transported to the plasma membrane where it fuse to release the mature virion from the infected cells to the extracellular space.

3. Epidemiology, pathogenesis and management of CMV disease

CMV disease is the result from a complex interplay between the consequences of the lytic viral replication, including the resulting inflammation, and the immunological status of the host. Primary CMV infection in immunocompetent host is sometimes

accompanied by clinical symptoms, but often is a silent infection. In immunocompromised host HCMV infections are a major cause of morbidity and mortality. In particular, CMV disease resulting from a primary infection in subject without prior specific immunity against HCMV may produce a more severe disease as opposed to infection resulting from reactivation of the latent infection or reinfection by a new virus strain. HCMV infection has also been associated with chronic disease, in both immunocompromised and immunocompetent hosts.

3.1 Mode of transmission and epidemiology

HCMV is a ubiquitous, opportunistic but usually asymptomatic pathogen in the whole population throughout the world. The seroprevalence is inversely correlated with the socio-economic conditions and reaches nearly 100% in children and adults from undeveloped countries and is estimated to be between 40 and 60% in adults of Northern America, Europe or Australia [29].

Although HCMV is a highly labile pathogen, it is readily transmitted during primary infection, reactivation or reinfection, by direct or indirect person-to-person contact. The first main period for natural transmission occurs during the perinatal period (0-2 years) and the young childhood [30]. Intrauterine infection in the United State is estimated between 0.1 and 1% of all live births (over 40,000 a year). In addition, horizontal mode of transmission to neonates includes contact with infectious genital secretions during birth (virus shed from the uterine cervix) and contamination via breast milk or saliva of the mother. A major route for HCMV transmission results from young children attending to group cares since HCMV infection during early years is associated with prolonged excretion of infectious virus into body fluid (saliva, urine) persisting for years. This plays a a major role for HCMV transmission to the parents.

The second main period of infection arise with sexual activity in adolescent and adults. HCMV sexual transmission follows close contact of oral or genital mucosa, HCMV being shredded by both male and female genital tracts. Seroprevalence reach 40% by adolescence and the infectious rate is estimated to increase by around 1% per annum.

3.2 CMV infections in normal hosts and aspects of latency

Although the primary infection is not associated with clinical symptoms in the large majority of normal host, it rarely manifests as nonspecific mononucleosis-like syndrome with fever, fatigue, atypical lymphocytes and evidence of mild hepatocellular damage (Table 1) [31, 32].

The course of HCMV dissemination usually begins at the mucosal epithelium site of the inoculation where the virus will easily replicate locally. Cell-to-cell transmission and lytic replication results in primary viremia. The virus further spreads to the main visceral sites of amplification e.g. the liver, lung and spleen [33]. Due to a broad cellular tropism, HCMV can infect almost every organ. HCMV dissemination is believed to be highly cell-associated, with EC playing a major role in this process [34]. A ubiquitous distribution of HCMV-infected EC have been detected in both microvascular and macrovascular vessels of various organs and have been shown to support productive lytic replication [9]. The expression of pro-inflammatory mediators (IL-8 and IL-6) in response to the endothelial layer infection will allow the recruitment of neutrophils and monocytes [35-38]. Both cell types will uptake virus particles during transmigration through the EC layer and transmit the infection into tissues. Whereas neutrophils do not support full HCMV replication and only passively transport infectious particles [39, 40], monocytes support the full replicative cycle once differentiated into tissue-resident macrophages and can release progenies into the corresponding organ [41]. In addition, neutrophils and monocytes may play a role in the establishment of latency.

Indeed, like all herpesvirus, HCMV establishes a lifelong latent infection in its host. Important mechanisms of latency remain to be elucidated: the exact nature of the latent state, the process of induction of latency or the reactivation from the latent state. Any organ from a seropositive donor was presume to vehicle latent CMV infection after the seroconversion of seronegative recipients of a kidney, heart, liver or lung graft from seropositive donors, despite the absence of any replicating virus found within culture of the graft biopsy [42]. In 1997, Hendrix et al. found HCMV DNA was widely distributed in organs of healthy subjects, confirming the hypothesis.

HCMV infection has been reported to highly impact a variety of EC functions *in vitro* and *in vivo.* Data from rodent studies emphasized an active role of CMV in vascular injury most likely through the induction of inflammation, however the exact mechanisms

remain to be defined [43, 44]. A study from Khoretonenko et al., performed in mice, showed that persistent CMV infection resulted in impaired endothelium function and mild inflammation in venules [45]. Moreover, *in vitro* infection of human EC with a clinical isolates or laboratory strain of HCMV elicited a potent inflammatory response characterized by the alteration of adhesion molecule expression or the up-regulation of pro-inflammatory cytokines [46-48]. Consistently, a growing number of epidemiologic studies have implicated HCMV in the pathogenesis of cardiovascular diseases (CVD), such as atherosclerosis or coronary artery disease [49-52]. HCMV-seropositive individuals without previous history of CVD present endothelial dysfunction and an increased atherosclerotic burden [53].

3.3 CMV disease in immunocompromised hosts

Neonates, HIV-infected patients and allografts recipients represent the three groups of immunocompromised patients of considerable medical importance associated with end-organ disease.

3.3.1 Congenital and neonatal infection

Congenital and neonatal infection with HCMV not only represents one of the leading causes of cognitive disorders in children, but is also a major cause of acute morbidity and mortality in neonates.

Mother to newborn HCMV transmission proceeds through three possible routes: transplacental resulting in congenital infection, perinatal or breastfeeding. These modes of vertical transmission correspond to 10 to 50% of infants with HCMV infection, in most cases asymptomatic. It is however to be noted that theses perinatally infected infants will persistently excrete large amounts of virus (up to 4-5 logs per ml of urine), sometimes for years, playing a major role as reservoirs for the spreading of CMV in the population. HCMV transmission to the fetus mainly occurs in case of maternal primary infection during pregnancy (transmission rate of 20 to 50%) as compared to women with preconceptional immunity (0.1-2.0%). Nevertheless, secondary maternal infection (reactivation or reinfection) is associated with a much higher incidence of long-lasting damage following congenital infection, although only 5% of infected infants show clinical symptoms at birth [33].

Intrauterine transmission is traditionally thought to rise from a viremic spread to the uterine-placental junction, ECs and the placental trophoblasts offering ideal

compartments for viral replication and entry of the virus in the fetal blood system. Damage to the fetus might occur in a variety of organ systems presumably by lytic replication (hepatobiliary system, CNS, lungs, hematopoietic system) [54]. The gestational age of the fetus at the time of infection seems to be of great importance regarding the extent and the severity of the disease (Table 1) and mortality to issue in around 10% of symptomatic newborns. Long-lasting sequel appears to results mainly in organ system with low regenerative capacity, brain damage and hearing loss being the most common manifestations occurring in 5% to 20% of cases.

Population	**Diseases associated with acute infection**	**Diseases associated with chronic infection**
Immunocompetent	Asymptomatic infection mononucleosis-like illness	Atherosclerotic vascular disease; inflammatory bowel disease, periodontal disease, rheumatologic disorders
Immunocompromised		
Fetal and newborn infants (congenital infection)	Asymptomatic infection (90%) 10% can have hepatitis retinitis, thrombocytopenia, neurologic disease	Hearing loss (5%-15%); neuron-development abnormalities
Allograft recipients	Fever, decreased bone marrow function hepatitis, pneumonitis bacterial superinfections	Transplant Vascular Sclerosis interstitial tubulosclerosis (renal allografts); loss of bile ducts (liver allografts) chronic graft rejection and graft loss
Immunodeficiency syndromes (acquired and inherited)	Retinitis, gastrointestinal disease (esophagitis, colitis), encephalitis	
Aged	Unknown	Immune senescence with potential decrease in immune responsiveness

Table 1: Clinical manifestations of HCMV infection

Adapted from W. Britt, Manifestations of HCMV infection; Current Topics in Microbiology and Immunology 325, 2008 [33]

3.3.2 Patients infected with HIV

HCMV is the most common and severe viral opportunistic infection in HIV infected patients. CMV disease is classified as an AIDS-defining illness according to the Center for Disease Control and Prevention [33].

Large prospective studies have associated increasing HCMV viremia as a strong predictor of the future development of CMV disease and death, independently of the $CD4^+$ cell count [55]. Over 90% of HIV patients are seropositive for HCMV. The loss of adaptive immune responses in the late stage of the disease increases the risk for acute HCMV infection and subsequent end-organ disease secondary to reactivation, uncontrolled virus replication and widespread viral dissemination. CMV disease was reported in 20 to 40% of AIDS patients [56].

The clinical manifestations of CMV disease and the organs involved are function of the severity of the immunological deficiency (Table 1). The most common and characteristic manifestations are CMV retinitis (80% of the patients) that can lead to loss of sight, and CMV colitis resulting from infection of the gastrointestinal tract (15% of the patients)[57]. The use of antiviral therapy, either local or systemic, with an effective inhibition of the virus replication can halt disease progression and lead to the resolution of the acute symptoms of the disease. The introduction of highly active antiretroviral therapy (HAART) resulting in the immune reconstitution and the rise of $CD4^+$ count has strongly decreased the occurrence of both CMV retinitis and colitis [58-60]. An unusual characteristic of CMV disease in adults is the involvement of the central nervous system giving rise to CMV polyradiculopathy and CMV encephalitis. These pathologies induce some cognitive and motor disturbances due to infection of the cerebrospinal fluids or the acute inflammation of the brain and were often fatal.

3.3.3 CMV disease in allografts recipients

HCMV is the single most important cause of infectious disease after solid organ transplantation (SOT). Over 75% of SOT recipients have laboratory and/or clinical evidence of CMV replication during the 1^{st} year post-transplant [61]. CMV infection has been associated with increased mortality and morbidity after transplantation and with diminished graft survival [62, 63]. The incidence of CMV disease varies according to the organ transplanted, the serostatus of the donor and the recipient and the level of immunosuppression.

HCMV infection in SOT recipients has been strongly correlated with reactivation-reinfection from a latent infection. Thus the seropositivity of the organ donors and recipients are used to define the risk of developing HCMV infection/disease in the post-transplant period. CMV seronegative patients receiving an organ from a CMV seropositive donor, D^+/R^- mismatch, have the greatest risk for infection: the incidence of symptomatic disease reaches 70%, with severe infection as a result of uncontrolled virus replication particular in the early post-transplant period [64]. These patients have about a two- to three-fold higher incidence of late disease following antiviral prophylaxis as compared to the other groups [65-67]. The combinations D^+/R^+ and D^-/R^+ are considered as intermediate risks for severe disease (10-30%) suggesting a predominant role of the pre-existing immunity in the control of HCMV replication. The nature of immunosuppressive regimen will have a major impact in these groups since the MHC-restricted virus-specific cytotoxic T cells are key players in the host defense against CMV[64, 68]. D^-/R^- have a relative low risk if CMV infection (5%). In the case of bone marrow transplantation, it is to be noted that the higher risk of infection occurs in D^-/R^+ especially when T-cell depletion is used.

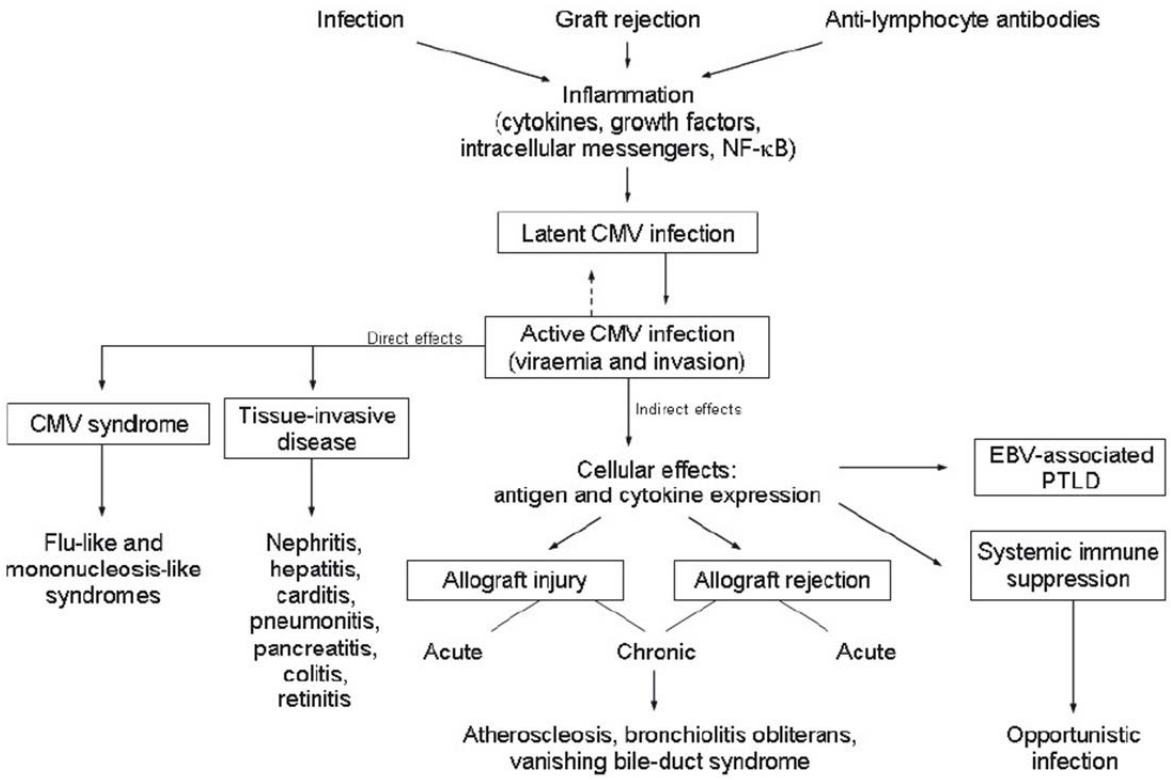

Figure 4: Overview of direct and indirect effects of CMV infection in allograft recipients.

Reproduced from Fishman et al., Clin Transplant, 2007[69]

HCMV replication or seroconversion commonly occurs within 3 months post-transplant in the absence of prophylaxis, and must not necessarily results in clinical symptoms (Table 1). The most common manifestation, the "CMV syndrome", is similar to any systemic viral infection and characterized by unexplained fever, flu-like or mononucleosis-like symptoms (including a 5-10% atypical lymphocytosis). Tissue-invasive disease occurs with a predilection for the greatest inflammatory pathology within the transplanted organ (hepatitis, pneumocystis and myocarditis) and can results in the destruction of the infected tissue either directly because of viral replication and cell lysis or as consequences of the host immune responses [61, 70].

Aside from the direct effects of infection, HCMV is associated with "indirect effects" in SOT recipients, mainly related to immune responses in the setting of longer periods of low-level viral replication (Figure 4). HCMV infection strongly contributes to a general non-specific immunosuppressive syndrome in transplant recipients, making the patients more susceptible to others opportunistic pathogens [64]. HCMV in associated with leukopenia (decreased number of polymorphonuclear leukocytes), a decreased ratio of T helper/ T cytotoxic ratio and an impaired cell-mediated immunity. Opportunistic infections, such as bacterial infections or accelerated hepatitis C after liver transplantation have been associated to SOT recipients with HCMV infection [71]. Co-infection with EBV increased by seven- to ten-fold the incidence of EBV-associated post-transplant lympho-proliferative disease [72, 73].

Although already hypothesized in 1970, a clear association between HCMV infection, allograft injury and rejection was only recently demonstrated with clinical studies using prophylaxis treatment. Renal transplants with D^+/R^- mismatch receiving anti-CMV prophylaxis showed a 50% reduction in organ rejection compared to placebo [74]. Similarly, anti-CMV prophylaxis improved liver transplant recipient's survival [75]. One clear manifestation of CMV-mediated allograft injury was reported in heart transplant recipients where both acute rejection and accelerated coronary atherosclerosis were identified in patients with symptomatic and asymptomatic CMV infection, coronary artery disease being a major cause of long-term mortality following transplantation [76]. The exact mechanism of CMV-induced allograft damage is unknown but is strongly believed to involve inflammatory changes, largely extend in presence of CMV in all forms of organs transplantation. Other factors such as ischemia reperfusion injury might be involved in these processes.

4. Management of HCMV infection

4.1 Prevention and treatment of CMV disease in transplant recipients

Different strategies are used in the transplant setting according to the group of risk aiming to reduce and maintain HMCV replication below a critical threshold and limit the chances of CMV disease development. Three strategies of administration are currently used: prophylactic, pre-emptive and therapeutic.

Universal prophylaxis consists in the administration of drug to an entire population, before any sign of active replication of the virus. Prophylaxis is usually given over the period of main immunosuppression (~100 days) with the goal to suppress viremia in the initial month following transplantation. In pre-emptive therapy, patients receive antiviral treatment when they are at particular risk for clinically important disease as shown by a particular laboratory or clinical/epidemiologic finding indicating a risk of CMV infection. This requires serial monitoring of population at higher risk with sensitive and rapid detection methods (via DNAemia by PCR in blood or pp65 antigenaemia).

Similar reduction of CMV-associated morbidity and mortality were shown with both strategies, which are currently recommended by guidelines [77, 78]. However, some concerns remains regarding the prevention of CMV disease. Although pre-emptive therapy reduces the exposure of patients to the risks of side effects associated with long-term drug treatment, it may not prevent low-level asymptomatic viral replication with the risk of indirect effects of CMV infection [79-81]. In a recent prospective cohort study of including over 95% of SOT recipients in Switzerland (1239 SOT patients between 2008 and 2011), a 6% incidence of CMV disease was reported after a median follow-up of 1 year. Although the incidence was similar regarding the antiviral preventive strategy, prophylaxis was shown to delay the CMV event after the period of treatment, whereas pre-emptive therapy was associated with an inferior transplant outcomes suggesting asymptomatic viremia early after transplantation [82].

Despite the use of prophylaxis or pre-emptive therapy, “late-onset CMV disease” resulting from virus reactivation within the 1st year following the antiviral therapy period still occurs in approximately 20 to 30% of patients at risk [83]. The typical course of treatment in this case is 3-4 weeks of acute therapy until all evidence of virus

replication is eliminated, clinical and laboratory improvement, and followed by 2-4 months of secondary prophylaxis [64].

4.2 Antiviral drugs

Three antiviral agents are currently approved for the systemic treatment of CMV infection and all belong to the nucleoside class of compounds: ganciclovir (GCV), cidofovir (CDV) and foscarnet (FOS). Formivirsen, an anti-sense RNA, is approved for the local treatment of CMV retinitis. To date no anti-CMV agent has been approved for the management of congenital CMV disease.

4.2.1 Ganciclovir

First specific antiviral agent approved for the treatment of CMV disease (1989 for CMV retinitis), GCV is the first-line choice for the treatment of CMV infection in the majority of clinical indications [84, 85]. Acyclic nucleoside analogue of 2'-deoxyguanosine, GCV is a prodrug that is required to be tryphosphorylated to be active, first by the CMV-encoded pUL97 protein kinase, then by the cellular enzymes (Figure 5). GCV triphosphate acts as competitive inhibitor of the viral DNA polymerase (pUL54) resulting in a slower chain elongation once incorporated in place of dGTP [86, 87]. In 1994, an oral formulation was introduced in addition to the original intravenous (IV) formulation (Cytovene-IV®, Roche), for prophylaxis and maintenance therapy. However, the low bioavailability (approximately 6%) and the high pill burden of this formulation were increasing the risk of lower systemic exposure and the emergence of drug resistance. In 2000, valganciclovir (an l-valyl ester of GCV) was approved with an oral bioavailability around 60% achieving a similar systemic drug exposure than the IV formulation with a once-daily administration. The major side effect associated with GCV therapy is neutropenia, which can be dose limiting and may be a risk for opportunistic infections. A risk of renal toxicity may also occur in combination administration with other nephrotoxic compounds (cyclosporine A) [88].

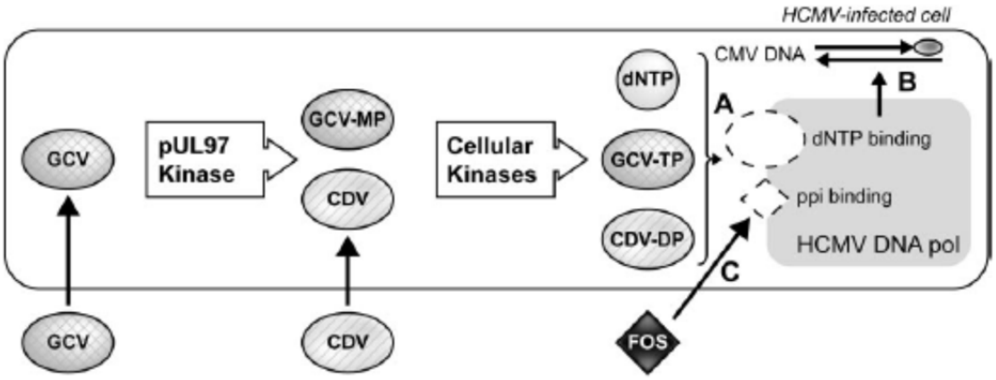

Figure 5: Mechanisms of action of systemic antiviral agents approved for treatment of CMV infection.

Reproduced from Gilbert and Boivin, AAC, 2005[89]

4.2.2 Cidofovir

Cidofovir (Vistide®, Gilead) is a phosphonomethoxy analogue of cytosine with a broad-spectrum antiviral activity against herpesviruses but also other DNA viruses (small pox virus) [90]. Although sharing the mechanism of action of GCV as a competitive inhibitor of the viral DNA polymerase, CDV carries a phosphate group and is thus converted in his active form by the cellular enzymes only (Figure 5). Marketed in 1996, CDV is only available in IV formulation and used as a second-line treatment only, mainly because of the great risk of severe nephrotoxicity [91]. CDV is also associated with neutropenia.

4.2.3 Foscarnet

Second drug approved for treatment of CMV retinitis in 1991, Foscarnet (Foscavir®, AstraZeneca) is a trisodium salt of phosphormophonic acid. Contrary to GCV or CDV, FOS does not require chemical modification for antiviral activation (Figure 5). It binds to the pyrophosphate-binding site of the viral DNA polymerase and blocks the cleavage of the pyrophosphate group from the terminal nucleoside triphosphate added to the growing DNA chain [92]. Only available in IV formulation as well, FOS is used as second-line therapy for patients who failed GCV therapy due to viral resistance or to dose-limiting neutropenia or leukopenia [85]. However, due to the risk of renal impairment, FOS administration requires slow infusion, extensive prehydratation and frequent monitoring of serum creatinine levels. [93]

4.2.4 Formivirsen

Formivirsen (Vitravene®, Isis Pharmaceuticals) is a 21-nucleotide anti-sense RNA targeting specifically the major IE transcriptional unit. Approved in 1998 as a second-line treatment of CMV retinitis in AIDS patients, Formivirsen is administrated by intraocular injection [94].

4.2.5 Compounds in development

Two antiviral compounds are currently in clinical development for the management and the prevention of HCMV infection.

Maribavir is an UL97 kinase inhibitor, shown *in vitro* to inhibit viral DNA assembly and inhibits egress of viral capsids from the nucleus of infected cells. Despites a good oral bioavailability, low host cell toxicity, maribavir failed as prophylaxis to prevent CMV disease in transplant patients [95, 96]. However, following recent positive outcomes of the management with maribavir of 12 patients with resistant CMV infection [97], additional phase 2 studies are ongoing in hematopoietic stem cell or SOT recipients.

Letermovir (AIC246) belongs to a novel class of low-molecular-weight compounds with strong *in vitro* anti-CMV activity in laboratory strains, clinical isolates or different resistant strain to approved drugs. Letermovir interferes with the UL56 subunit of the viral terminase complex blocking the viral DNA cleavage/packaging in the late phase of the replication cycle [98]. Favorable outcomes were reported in a phase 2a clinical study for the use of letermovir as preemptive therapy and in a phase 2b study for HCMV prophylaxis in stem cell transplant recipients [99].

4.2.6 Resistance to antiviral drugs

Drug-resistant CMV infection was first seen in patients with AIDS. Prior to the introduction of HAART, several studies reported clinical isolates from AIDS patients with GCV- resistance with an incidence of 2-7% after 2-3 months of therapy and 15-28% after 9 or 18 months [88, 89]. HCMV resistance is today an emerging problem in SOT recipients. The incidence and temporal emergence of GCV resistance varies greatly according to the type of transplant, the different strategies of treatment and the immunosuppressive regimen used. In a retrospective study of 240 SOT recipients, GCV-resistance rates occurs in 2.1% of all patients and in 7% of D^+/R^- subjects (67 out of 240), with the highest frequency in kidney-pancreas or pancreas recipients (21%) and kidney recipients (5%) [89].

HCMV resistance to GCV is associated with mutations mapped to both UL54 (viral DNA polymerase) and UL97 (protein kinase) genes [100], UL97 mutations been identified by genotypic studies in over 90% of GCV-resistant HCMV clinical isolates [101]. These point mutations or small deletions were located in the codons 460, 520 or in the cluster 590-607 of the UL97 genes, resulting in impaired drug phosphorylation but without preventing the protein kinase function [89]. Being the only drug requiring a monophosphorylation, theses mutations do not affect the antiviral potential of the other nucleoside analogues. In contrast, mutations in the pol gene UL54 generally occur in specific conserved subdomains and may confer cross-resistance to CDV or less commonly FOS [102]. However, several clinical isolates were shown to have mutations in both the UL54 and the UL97 genes and to be cross-resistant to GCV, CDV and/or FOS [103].

Altogether, risk factors for the emergence of GCV resistance include suboptimal plasma or tissue drug concentration, a sustained and profound immunosuppression status, a high HCMV load, frequent episodes of HCMV disease and frequent discontinuation of the treatment due to toxicity. In addition, the lack of HCMV-specific immunity (D^+/R^- group) is a major risk factor in SOT.

II. Statins: not just a family of cholesterol lowering drug

Cardiovascular diseases (CVD) are the principal cause of mortality in developed countries. An estimated 17.1 million people died from CVD in 2004, representing 29% of all global deaths (WHO, Fact sheet 317, January 2011). Elevated level of serum cholesterol or hypercholesterolemia (HC) is one major risk factors modifiable for CVD prevention. A strong association has been established between HC and increased atherosclerotic disease, the underlying disorder in the majority of patients [104]. Statins, a family of potent inhibitors of the cholesterol biosynthesis pathway, is the most commonly prescribed treatment for HC. Interestingly, statin therapy appears to promote additional effects that are independent from changes in serum cholesterol level. Among them, antiviral activity against several viruses has been reported.

1. Cardiovascular disease and cholesterol lowering drugs

1.1 Cardiovascular risk factors

Running from 1948 to 1971, the Framingham Heart Study was the first large-scale study to evaluate the causes of atherosclerotic heart disease. Several main risks factors were defined which cannot be influenced (age, gender, heredity) but some are adjustable, such as high blood pressure, HC, smoking, obesity or diabetes [104-108]. The level of total cholesterol in the blood and in particular the ratio between high-density and low-density lipoprotein (LDL) is now considered a strong predictor of the likelihood to develop coronary heart disease. Cholesterol is an essential component of cell membranes, the source being by one-third food and by two-thirds synthesis in the liver. Thus, diet alone may not result in a great enough reduction in cholesterol levels and may need to be combined with cholesterol-lowering drugs.

1.2 Statins introduction and clinical benefits

The first identification of a fungal metabolite (compactin) blocking cholesterol synthesis was done in 1971 by Endo et *al* [109]. Although its administration to rats resulted in significant inhibition of cholesterol biosynthesis, the unacceptable hepatocellular toxicity made any further clinical development impossible. In 1978, a new metabolite

called lovastatin was isolated from *Aspergillus terreus* and displayed a more potent inhibition of the 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, the enzyme catalyzing the rate-limiting step of the cholesterol biosynthesis pathway (Figure 7). Without hepatocellular toxicity when administrated in rats, lovastatin became the first of its class of cholesterol-lowering agents to be approved for clinical use in human [110]. Since then, three generations of statins, both natural and chemically modified, have been commercially introduced: pravastatin and fluvastatin (first generation), atorvastatin and simvastatin (second generation), and finally rosuvastatin and pitavastatin (third generation) [111]. Although all statins share an HMG-like moiety and competitively inhibit the HMG-CoA reductase by similar mechanism, structural adaptations were used to improve pharmacokinetics, drug-drug interactions and safety [112].

Since introduction into the marketplace in 1986, statins have become one of the main medications thanks to their efficient prevention of both primary and secondary CVD [113-118]. Interestingly, two mechanisms were shown to be involved into statin-mediated reduction of serum cholesterol levels: the reduction of endogenous cholesterol synthesis via the HMG-CoA reductase inhibition and the increase of cholesterol clearance from the bloodstream via the increases in hepatic LDL-containing cholesterol receptor activity [119]. Clinical data reporting a decrease in cardiovascular events and regression of atherosclerosis, especially following early initiation of statins, strongly suggested statins also exert some beneficial effects beyond their ability to lower cholesterol. In 2006, the ARMYDA study reported a strong reduction of myocardial infarction during or shortly after coronary intervention as well as a concomitant attenuation of post-procedural endothelial inflammatory response [120, 121].

This anti-inflammatory activity of statins is supported by other studies (PRINCE, PROVE-IT/TIMI22, REVERSAL, MIRACL) where statin therapy resulted in a reduced risk of acute coronary syndromes in patients with normal LDL cholesterol (<3.4 mmol/L) but increased C-reactive protein (CRP) concentration, a marker of vascular inflammation [122, 123]. The JUPITER study, a 4-year trial designed to assess the benefit of statin therapy in patients with increased inflammatory response (defined by a high-sensitivity CRP level) but with normal LDL-cholesterol level, provided the strongest evidences of the beneficial use of statins in primary prevention of cardiovascular risks [124]. The study was discontinued after 1.9 years only, having reached a significant 47% reduction

of various cardiovascular events in the group of interest, over 17 802 men and women recruited and randomized for rosuvastatin or placebo treatment.

1.3 Atherosclerosis, an ongoing inflammatory disease

The understanding of statin therapy benefits in prevention of CVD is related to the pathogenesis of atherosclerosis. Atherosclerosis is a complex, slow process, chronic inflammatory response of arterial blood vessels, resulting in accumulation of cholesterol-dense macrophages, smooth muscles cells proliferation and the elaboration of multiple plaques, leading to arteries thickening with the risk of thrombosis [125].

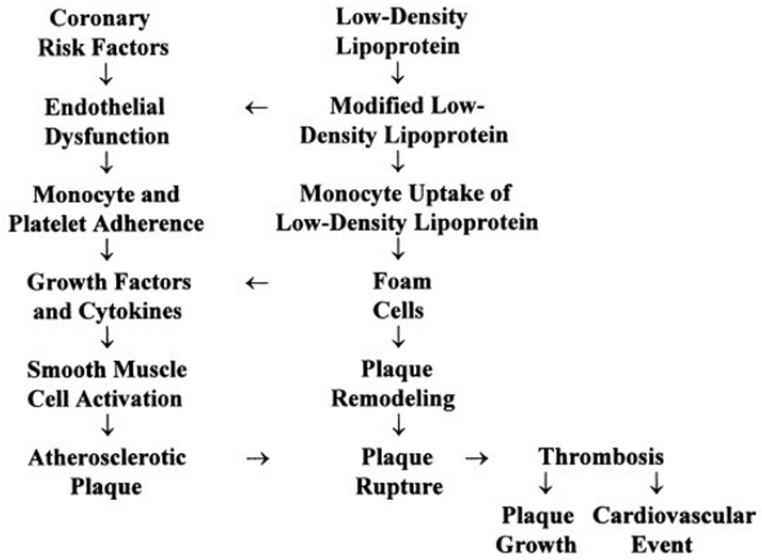

Figure 6: Key steps in the development of atherosclerosis

Reproduced from R. Vogel, Am J Med, 1999[126]

The disease is thought to be initiated by a complex interplay between endothelial dysfunction and lipids accumulation and modification (Figure 6). Cardiovascular risk factors tend to perturbate the endothelium main role of regulation of the vessel tone in response to physical and humoral conditions, via the secretion of several biologically active species with autocrine, paracrine and endocrine metabolism. In particular, oxidized LDL cholesterol stimulates vascular cells to produce inflammatory cytokines and recruit leukocytes [126-128]. After adhering to the activated endothelial monolayer, monocytes migrate into the intima and mature into macrophages, contributing to lipid uptake and finally turning into foam cells. The lesion progression involves the migration of vascular smooth muscle cells (VSMC) from the media to the intima and the synthesis of extracellular matrix macromolecules. Extracellular lipid derived from dead or dying cells (macrophages and VSMC) can accumulate in the central region of a plaque, often

denoted the lipid or necrotic core, together with cholesterol crystals and microvessels. The ultimate complication of atherosclerosis occurs in case of rupture of the plaque, causing thrombosis and leading to acute ischemic episodes in both cardiac and peripheral vascular systems [129].

1.4 Clinical manifestations and molecular biology of statins pleiotropic effects

Statins have been introduced as inhibitors of the HMG-coA reductase to lower LDL-cholesterol synthesis and serum levels. However, this enzyme is catalyzing the rate-limiting step of the mevalonate biosynthetic pathway, a complex biochemical pathway generating several fundamental end-products, including cholesterol, isoprenoids, dolichol (protein glycosylation), ubiquinone (respiratory chain activity), and isopentenyladenine (Figure 7) [130].

Thus, by inhibiting the synthesis of mevalonate, statins also prevent the synthesis of essential isoprenoids intermediates of such as the farnesyl and geranylgeranyl pyrophosphate (FPP and GGPP, respectively) that are required for the post-translational modification (isoprenylation) of various small GTP-binding proteins including Ras, Rap, Rab, Rho, nuclear lamins, and the heterotrimeric G-protein c-subunit [119, 131, 132]. Isoprenylation is needed for anchoring these signaling proteins to the cellular membranes in close proximity to growth factor receptors, to be activated following receptor activation, and subsequently participate in turning on downstream mitogenic signaling pathways such as MAPK and Akt [133]. Ras farnesylation was shown to induce VSMC migration and proliferation, Rho GTPases to be involved in the regulation of the actin cytoskeleton, the cellular migration, the cell division and adhesion, playing a major role in vascular structure and function [134, 135]. Thus, by targeting the HMG-CoA reductase, statins not only block cholesterol biosynthesis, but also affect key proteins involved in vascular inflammation and remodeling [136-138]. These cholesterol-independent effects or so-called “pleiotropic” effects are not limited to endothelial cells. Indeed, statins being mostly lipophilic are considered to enter cells by passive diffusion through the cell membrane and to affect multiple cell populations relevant to both inflammatory and immune responses, including B cells, T cells, regulatory T cells, macrophages, dendritic cells or platelets [139].

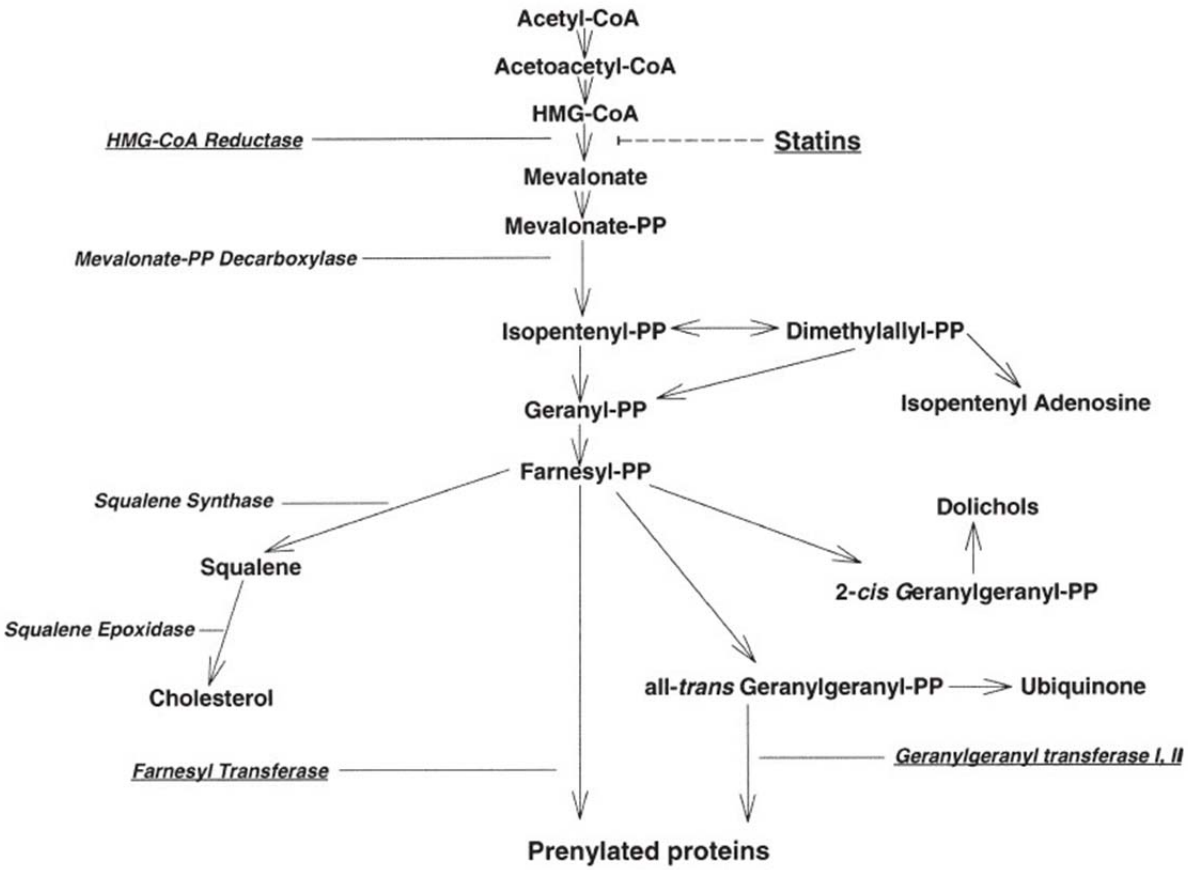

Figure 7: The mevalonate pathway

Reproduced from A. Corsini et al., Pharmacology & Therapeutics, 1999 [130]

The reduction of vascular inflammation is likely to be an essential modus operandi for statins to exert vasculoprotective effects [138, 140, 141]. Statins were shown to reduce the amount of inflammatory cells in atherosclerotic plaques [142]. Indeed, the inhibition of the expression of the adhesion molecules and the major histocompatibility complex class II by statin was demonstrated in EC and monocytes [143-145]. One important characteristic of endothelial dysfunction is the decreased synthesis and activity of endothelial-derived nitric oxide (eNOs), a major inhibitor of the atherogenic process in thus that it mediates vasodilatation, inhibits platelet aggregation and VSMC proliferation and prevents monocyte adhesion [146]. eNOs, which expression is reduced in proatherogenic conditions (hypoxia, oxydised-LDL-C or cytokines), is upregulated by statins who increased eNOs mRNA stability through the inhibition of RhoA [147]. A third mechanism recently revealed might also be involved in these immune-modulatory effects. Lovastatin and other statins were shown to specifically bind to an allosteric site of the leukocyte function-associated antigen-1 (LFA-1) resulting in inhibition of LFA-1 binding to intracellular adhesion molecule (ICAM-1). Interestingly, this selective inhibition is unrelated to the inhibition of HMG-CoA reductase [148, 149].

Altogether, the therapeutic effects of statins are a combination of cholesterol-lowering and cholesterol-independent effects at both molecular and cellular levels resulting in wide-ranging clinical benefits in CVD prevention, including improvement or restoration of the endothelial function, enhancement of the atherosclerotic plaques stability and decrease of vascular inflammation.

1.5 Antiviral activity of statins

Interestingly, statins were also reported to exert beneficial properties against a wide-range of pathogens. The mechanisms of the *in vitro* antiviral activities of statins against several viruses are resumed in Table 2.

Obviously, cholesterol reduction is the first mode of action through which statins might impair viral infections. Cholesterol is an essential component of cellular membranes and cholesterol depletion could thus interfere with several steps of the virus replication cycle. Statin-mediated reduction of cellular cholesterol *in vitro* was shown to affect the replication of pseudorabies virus, poliovirus or rotavirus [150, 151]. The major role played by prenylated proteins into intracellular signaling pathways explained the antiviral activity of statins against the respiratory syncytial virus, the hepatitis C virus (HCV) replication and HIV-1. Interestingly, different mechanisms were reported in HIV-1 inhibition, including the inhibition of LFA-1 binding to ICAM-1.

In vivo evaluation of anti-HIV potential of statins revealed a decrease of the viral load and increase of the $CD4^+$ cell counts after 1 week of treatment in an acute model of infection in SCID mice grafted with adult human peripheral blood mononuclear cells [152]. However, the clinical confirmations of these data are controversial. In a proof-of-concept small-scale study, del Real *et al.* reported a net reduction of viral RNA loads and in general, an increase of the $CD4^+$ T cell counts in six chronically HIV-1 patients not receiving HAART. The discontinuation of statin treatment was followed by a rebound in viral load. In contrast, 2 studies evaluating atorva- or simvastatin in HIV-1 patients after HAART discontinuation did not report any significant reduction in the viral loads or in the $CD4^+$ cell loss over 12 weeks of treatment, despite their ability to reduce patients' serum cholesterol levels [153, 154]. Finally, when patients with stable antiretroviral therapy were assessed, pravastatin did not exert a clear effect on viral load but was associated with substantial viral rebound in some patients after discontinuation of treatment [155]. Altogether, these studies do not support the uses of statins as antiviral

agents in HIV-infected patients outside of their use as cholesterol-lowering drugs. Moreover, significant drug-drug interactions have been reported between some statins and protease inhibitors exacerbating their inherent toxicities [156].

The anti-HCV activity of statins is probably the most studied one, alone or in combination with selective HCV inhibitors. *In vitro*, the potency of the different statins was evaluated. Atorvastatin, fluvastatin, mevastatin and simvastatin were reported to have similar anti-HCV activity. Lovastatin was shown to be less effective in inhibiting HCV replication and pravastatin not to exert any [157, 158]. The additive activity of most statins against HCV was demonstrated *in vitro* with IFN-α as well as with different specific HCV polymerase inhibitors and protease inhibitors. Moreover, drug-resistant HCV replicon exhibited similar susceptibility to mevastatin, and the combination with the non-nucleoside polymerase inhibitor HCV-796 delayed or prevented the emergence of resistant replicon. Clinical evaluation of statins on HCV is limited. Atorvastatin was investigated in a 12-weeks prospective trial in HCV-infected patients who required treatment for hypercholesterolemia. Despite the reduction of the serum cholesterol, no significant change in HCV RNA levels was reported [159]. In contrast, fluvastatin evaluated as monotherapy showed modest, variable and often short-lived suppressive effects on HCV viral loads in chronic HCV carriers [160]. Recently, rosuvastatin was reported to be beneficial in addition to IFN-α and ribavirin treatment in chronic HCV patients with nonalcoholic fatty liver disease and to significantly reduce viremia, steatosis, and fibrosis without causing side effects [161].

In 2003, Horne et al. evaluated the effect of statin therapy, as part of the normal medication, among CMV seropositive patients with angiographically significant coronary artery disease [162]. Statins reduced the increased mortality rates associated with CMV-seropositivity. The authors attributed these beneficial effects of statins to immune-altering, anti-inflammatory or antioxidant properties, rather than to a direct anti-viral effect. However, fluvastatin has been shown to restrain HCMV replication in human umbilical vein EC by inhibiting IE antigen expression and subsequent DNA synthesis [163]. A reduction of NF-κB binding activity by fluvastatin was suspected to limit the transactivation of the CMV-IE promotor.

Table 2: Antiviral activities of statins [150-152, 157, 158, 160, 164-172]

Virus	Statins	*In vitro / In vivo*	cholesterol depletion/ pleiotropic	Results / Mechanism	Viral target	Cellular target	Publication
Poliovirus *picornaviridae*	simvastatin	*in vitro*	cholesterol depletion	Impairement of the viral genome transcription	viral RNA transcription	Intracellular vesicles formation	S. Liu *et al.* *BBRC, 2006*
Rotavirus *reoviridae*	lovastatin	*in vitro*	cholesterol depletion	Defective viral particle assembly	ND	-	K.V. Mohan *et al.* *Arch Virol, 2008*
Pseudorabies virus *alphaherpesviridae*	lovastatin	*in vitro*	cholesterol depletion	Reduced infectivity and stability of progeny virus	-	-	A.S. Desplanques *et al.* *Virus Research, 2010*
Respiratory Syncytial Virus *paramyxoviridae*	lovastatin	*in vivo (mice)*	pleiotropic	Diminishes RSV replication and RSV-induced illness in mice	-	-	T.L. Gower *et al.* *AAC, 2001*
		in vitro		Inhibit RSV-mediated cell-to-cell fusion	fusion glycoprotein F	RhoA membrane localisation	
Dengue virus *flaviviridae*	lovastatin	*in vitro*	both	Impaired viral assembly process in the endoplasmic reticulum	-	Rab5 and Rab8 proteins ?	M. Martínez-Gutierrez *et al.* *Intervirology, 2011*
Hepatitis B virus *flaviviridae*	simvastatin	*in vitro*	ND	Effective inhibtion of HBV replication Potentiate all four licensed nucleosite analogues anti-HBV activity	ND	ND	T. Bader et al. *Antiviral Research, 2010*
Hepatitis C virus *flaviviridae*	lovastatin	*in vitro*	pleiotropic	Prevent the assembly of HCV replication complex	HCV NS5A non structural protein	FLB2 geranygeranylation	J. Ye et al., *PNAS, 2003* C. Wang et al. *Mol Cell ,2005*
	atorvastatin fluvastatin lovastatin pitavastatin simvastatin	*in vitro*	pleiotropic	Prevent HCV RNA replication with various potential: pita>flu>ato>sim>lov Enhance the anti-HCV activity of IFN-α	-	-	M. Ikeda et al. *Hepatology, 2006* *J Pharmacol Sci, 2007*
	mevastatin simvastatin	*in vitro*	pleiotropic	Additive antiviral effect with HCV polymerase and protease inhibitors Prevent the selection of drug-resistant HCV replicon	-	-	L. Delang et al. *Hepatology, 2009*
HIV-1 *retroviridae*	lovastatin	*in vivo (mice)*	pleiotropic	Decrease viral load and increase CD4+ cell counts in acute infection model	-	-	G. del Real et al. *J Exp Med, 2004*
		in vitro		Inhibition of virus entry and budding	ND	Rho GTPases (gp120-CXCR4 colocalisation?)	
	lovastatin	*in vitro*	pleiotropic	Diminish HIV-1 attachment to target cells	-	block ICAM-1-LFA-1 interactions	J.F. Giguère et al. *J Virol, 2004*
	lovastatin mevastatin simvastatin	*in vitro*	pleiotropic	Preferential reduction of CD4+ lymphocytes infection with R5 virus as compared to X4	-	decreased CCR5 cell surface expression increased secretion of RANTES	A.A: Nabatov et al. *Plos One, 2007*
	lovastatin simvastatin	*in vitro*	pleiotropic	Suppress HIV-1 virion release from TNF-α stimulated latently infected cells	-	Rab11a GTPase (facilitating intracellular vesicles traficking)	T. Amet et al. *J Mic Inf, 2008*

Aims of the study

In the absence of vaccines against HCMV and considering the drawbacks limiting the use of current antiviral drugs, there is an urgent need for new therapeutic approaches for the management of acute HCMV disease in immunocompromised patients. Beyond anti-infective therapies, other medications may provide a means to treat infection-associated effects, especially the role played by HCMV into the pathogenesis of atherosclerosis and ultimately prevention of cardiovascular diseases. One effective strategy consists in looking for known drugs with a good safety profile that also affect viruses.

Statins are a well-tolerated and extensively studied group of cholesterol-lowering drugs, exhibiting strong anti-inflammatory and immune-modulatory activities as well as antiviral effects on different viruses alone or in combination with selective antiviral inhibitors. So far, despite the large number of statins currently used in clinic and their differences in term of tissue permeability and metabolism, only fluvastatin was tested for anti-CMV activity.

This study was aiming to:

 # 1. Explore the *in vitro* anti-cytomegalovirus activity of various statins in two relevant cell-types in HCMV infection, human endothelial cells and fibroblasts

 # 2. Provide insights into the cellular and molecular mechanisms of statins' anti-cytomegalovirus activity

 # 3. Evaluate the potential clinical benefits of the anti-cytomegalovirus activity of statins alone and in combination with ganciclovir

Materials and Methods

1. Cells

Primary human aortic endothelial cells (HAEC, Lonza® CC-2535) and cardiac microvascular endothelial cells (HMVEC-C, Lonza® CC-7030) were respectively used at passages 9 to 14 and 5 to 10, and were cultured in EGM-2 medium (Lonza) following the manufacturer's instructions.

The human fetal lung fibroblast MRC-5 (ATCC® CCL-171) at passages 30 to 45 were supplemented in Dulbecco's Modified Eagle Medium (DMEM, Invitrogen) containing 10% fetal calf serum (FCS, PAA laboratories ltd) and 2 mM L-glutamine (Invitrogen).

All cell preparations were tested negative for mycoplasmas by 4,6-diamidino-2-phenylindole staining (DAPI; Sigma).

2. Viruses

The HCMV endotheliotropic TB40/E and fibrotropic TB40/F strains were derived from a bone-marrow transplant recipient by 22 passages in EC and fibroblasts, respectively [173].

For preparation of virus stocks, MRC-5 were infected with TB40/E or TB40/F at a MOI of 0.5. Supernatants of infected cultures were harvested at 7 days post infection (dpi) and cleared of cellular debris by a 10 minute (min) centrifugation at 2,500 g. Virus stocks were partially purified by an ultracentrifugation step over a 15% sucrose cushion (50mM Tris-HCl, 12mM KCl, 5mM Na_2EDTA) at 20,000 rpm for 90 min at 4°C using a SS-34 rotor (Beckman Coulter). Virus aliquots were stored at -80°C. The infectious titer was determined by the Tissue Culture Infective Dose 50 ($TCID_{50}$) assays (see below) in MRC-5 seeded in 96-well plates [174]. All virus preparations were tested for mycoplasmas by DAPI staining.

The GCV-resistant strain pp6, derived from an AIDS patient and characterized with both M460I and M460V mutations of the UL97 gene was kindly provided by K. Hamprecht [175].

3. Reagents

Stock solutions of atorvastatin calcium salt (Molekula), fluvastatin sodium salt (Calbiochem), simvastatin and pravastatin sodium salt (Sigma) were done in dimethyl sulfoxide (Sigma). Mevalonolactone and geranylgeranyl pyrophosphate ammonium salt (Sigma) were dissolved in ethanol and methanol, respectively. Water soluble cholesterol, methyl beta cyclodextrin (Sigma) and ganciclovir (Cymevene®; Roche) were dissolved in water. IFN-α was purchased from Peprotech.

All stock solutions were stored according to manufacturer's specification until use. Drug dilutions were prepared extemporaneously in culture medium. Except for statins, all reagents were assessed by trypan blue exclusion and alamarBlue® assay, respectively, and used at subtoxic doses (data not shown). The final concentrations of all vehicles in media never exceeded 0.1% (v/v) and did not show any alteration of cell viability or cell proliferation.

Special precautions were taken to avoid exposure of statins to light, before and during incubation procedures.

4. Treatment and infection

For all experiments, cells were seeded at 25,000 cells/cm^2 for HAEC and 50,000 cells/cm^2 for MRC-5 in fresh culture medium in order to reach cell confluence at the time of treatment. Confluent monolayers were treated with statins and other drugs 24 hours prior to infection, except if specified otherwise. GCV was added 2 hours post infection.

To infect cells, medium was removed and put aside. Cells were washed with Dulbecco's Phosphate Buffer Saline (PBS, Lonza) and inoculated with virus diluted in RPMI 1640 (Invitrogen) at the indicated MOI for 90 min at 37°C. After inoculation, the virus dilution was removed, cells were washed with PBS and replaced by the original medium. Cells were kept in culture with drug-containing medium for the corresponding time.

5. Cell proliferation assay

Doses of statins used for this study were defined based on their anti-proliferative activity determined using the alamarBlue® assay (Invitrogen). Briefly, cells were plated in 96-well plates in triplicate at 2,000 cells per well and 4,000 cells per well for HAEC and MRC-5, respectively. Cells were exposed to serially diluted concentrations of statins

ranging from 0 to 1,000 µM, for a 72-hour incubation. Every day, the cell viability reagent was added to respective wells and absorbance at 570 nm was determined using a Victor2 Multilabel Plate Reader (Wallac, PerkinElmer) after a 4-hour incubation at 37°C.

6. Tissue Culture Infective Dose 50 assay

HCMV titers were determined in supernatants of infected cultures by the $TCID_{50}$ assay. Briefly, supernatants were harvested and cleared of cellular debris by centrifugation for 10 min at 2,500g, aliquoted and stored at -80°C. The infectious titer of each supernatant was determined in duplicate using distinct aliquots. Serial dilutions of supernatants were used in quadruplicates to infect 96-well plates of MRC-5 for a 24-hour period. Cells were then fixed with 80% acetone (Sigma) and stained with antibodies directed against the IE proteins IE72 and IE86 (pUL122/123; mouse-anti-human E13, Argene) and detected with the Cy3-conjugated secondary goat anti-mouse-IgG Fab'2 (Jackson ImmunoResearch). Stained cultures were examined for IE-positive cells with the fluorescent IX71 microscope (Olympus). Each well containing at least one IE-positive cell was defined as an infected culture. Infectious titers were calculated as described by Mahy & Kangro and expressed in $TCID_{50}\,mL^{-1}$.

7. qRT-PCR

Following isolation from cells and supernatants using Qiagen DNeasy Blood & Tissue Kit (Qiagen), HCMV DNA was amplified and quantified using specific primers and probes for a 62 bp region of the glycoprotein B gene (UL55) as previously described [176]. DNA quantification was performed in duplicate in a total volume of 30 µL containing 10 µL DNA and using HotStarTaq master mix (Qiagen) supplemented with PCR primers (1 µM each) and probes (0.3 µM) and additional $MgCl_2$ (1.5 µM). Amplification was performed in a Real-Time thermocycler (iQ5 Cycler; Bio-Rad) as follows: 13min 30s at 95°C and 50 cycles of 15s at 95°C and 60s at 60°C.

8. Western blot analysis

For immunoblotting, protein samples were prepared by lysis of infected cultures grown in 24 or 12-well plates in sodium dodecyl sulphate (SDS) sample buffer (50 mM tris–HCl, pH 6.8, 10% glycerol, 2% SDS, 10% β-mercapto-ethanol, and 0.1% bromophenol dye). Protein samples were separated by electrophoresis on a 10% polyacrilamide gel, transferred to a polyvinylidene difluoride membrane (Hybond-P, GE Healthcare). After

blocking with 5% non-fat dried milk (Bio-Rad) in 2% tween/tris buffer saline, membranes were incubated overnight with primary antibody at 4°C, extensively washed with 2% tween/tris buffer saline and followed by a 1-hour incubation with a horseradish peroxidase-conjugated secondary antibody (GE Healthcare, 1/5,000). The targeted proteins were revealed by enhanced chemiluminescence using the ECL™ western blotting detection reagents (GE Healthcare).

The following primary antibodies were used in this study: mouse anti-CMV IE72/IE86 (clone E13, Argene), mouse anti-CMV pp52 (clone 10D8, Virusys corporation), mouse anti-CMV pp65 (Virostat), mouse anti-human β-actin (clone AC-15, Sigma) and mouse monoclonal anti-myxovirus protein A antibody, kindly provided by J. Pavlovic [177].

9. Cellular cholesterol quantification

For cellular cholesterol quantification, cell extract samples were prepared by lysis of cultures grown in 12-well plates. Special attention was given to determine a proper lysis buffer (50mM Tris, 2mM CaCl2, 80mM NaCl, 1% Triton X-100) compatible with both cholesterol and protein quantification methods. Cholesterol quantification was performed using the Amplex® Red Cholesterol Assay Kit (Invitrogen), a sensitive fluometric method allowing the quantification of cholesterol and cholesterol ester by using a cholesterol standard curve. A total protein quantification was performed using the Pierce Bicinchoninic Acid (BCA) Protein Assay Kit (Thermo scientific), a detergent-compatible colorimetric method using an albumin standard curve for quantitation of total protein. Both assays were performed in 96 well microplate and read using the Synergy 2 multi-mode reader (Biotek).

Every sample was quantified in duplicate for cholesterol content and normalized with protein concentration.

10. Flow cytometry analysis

Cells grown in 6-well plates (200,000 cells/well) were incubated for 5 min with PBS-2nM EDTA, washed and stained for 30 min at 4°C with PE, APC or Alexa Fluor 488-conjugated monoclonal antibody (mAb) to PDGFRα, β1 integrins and EGFR, respectively. The PE or APC-conjugated MOPC21 (BD Pharmingen) and the Alexa Fluor 488 conjugated normal mouse IgG (Millipore) were used as isotype-matched controls. The fluorescence intensity was measured by using a FACSCanto (BD Biosciences) and analysis was performed using FlowJo software (Tree Star).

The following primary antibodies were used in this study: APC conjugated mouse anti-β1 integrins (Thermo Scientific for flow cytometry, clone MEM-101A), mouse anti-αV integrin (Santa Cruz, clone P2W7) and mouse anti-β3 integrin (Santa Cruz, clone BV4), PE conjugated mouse anti- PDGFRα (Santa Cruz clone 16A1 for flow cytometry), AF488 conjugated mouse anti-EGFR (Millipore for flow cytometry, clone LA1)

11. Cell cycle progression analysis

Cells seeded in 6-well plates (200,000 cells/well) in serum-free medium were treated for 1 or 4 days with statins at 37°C. Cells were then trypsinized, washed twice with cold PBS by resuspending and spinning down (2,000 rpm, 5min). After the last centrifugation, supernatant was discarded and cells were fixed with 70% ice-cold ethanol and incubated overnight at 4°C. The next day, cells were centrifuged 5 min at 3,000 rpm, the pellet was stained for 30 min at 37°C in the dark under gentle shacking with a staining solution (PBS, 0.3mg/ml of RNAseA, 0.3mg/ml of propidium iodide). After centrifugation (5 min, 3,000 rpm) cells were resuspended in 100µl of the staining solution and analyzed by flow cytometry within 24 hours. The fluorescence intensity was measured by using a FACS Canto (BD Biosciences) and analysis was performed using FlowJo software (Tree Star).

12. Statistical Analysis

All values are reported as mean values ± SD, the number of experiments is specified in the figure legends section. Statistical comparisons between experimental conditions were performed with the Stat View (V5.0.1) software using the analysis of variance followed by Fisher's exact test with statistically significant differences set as: *$p < 0.05$,**$p < 0.01$,***$p < 0.001$.

Fifty percent and ninety percent effective dose ($ED_{50,}$ ED_{90}) values were calculated using nonlinear regression curve fit with a variable slope. GraphPad Prism 5.04 software was used for all analyses.

Results

I. Statins exert an anti-cytomegalovirus activity in aortic endothelial cells

1. Anti-proliferative activity of statins on HAEC

Besides a shared HMG-like moiety allowing the competitive inhibition of the HMG-CoA reductase, statins differ in their potency for mevalonic acid synthesis inhibition mainly because of large differences of solubility, tissue permeability and metabolism, related to their chemical structures [147].

In order to compare the effect of four chemically-different statins on HCMV replication, we defined 3 doses for each of the statins: the IC_{50}, IC_{20} and sub-inhibitory dose (SD), being the concentrations responsible for 50%, 20% or no cell growth inhibition, respectively, as compared with untreated control cell growth. Increasing doses of atorva-, fluva-, prava- and simvastatin ranging from 0 to 1,000 µM were added to the cells 24 hours post seeding. Cell proliferation was quantified every 24 hours within a 72-hour period of time in an alamarBlue® assay.

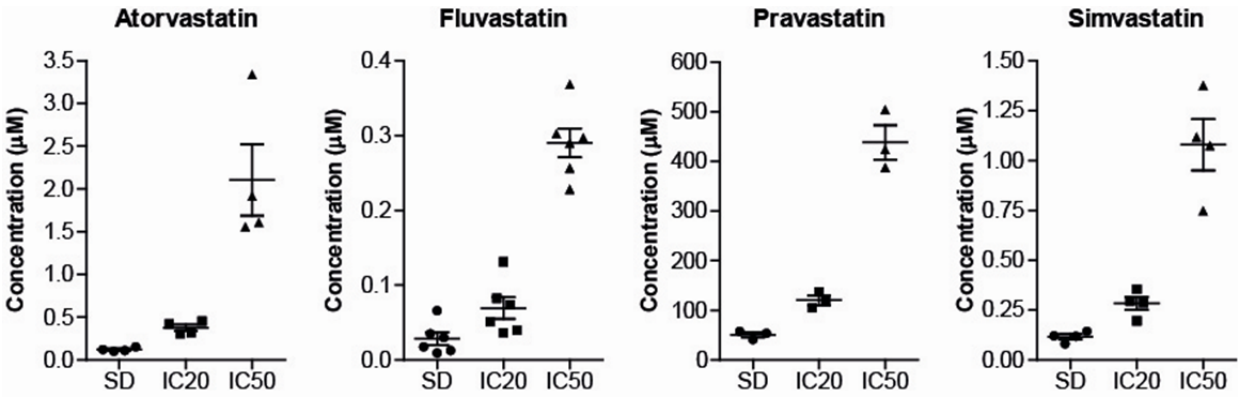

Figure 8: Anti-proliferative activity of statins in HAEC after a 72-hour drug exposure period.

Scatter plots of the statins concentrations responsible for 50% (IC_{50}), 20% (IC_{20}) or no cell growth inhibition (SD) as compared with untreated culture. Bar represent mean ± SEM of at least 3 experiments.

Whereas atorva-, fluva- and simvastatin exhibited a fairly comparable toxicity on HAEC, pravastatin was drastically less toxic (Figure 8). The SD of atorva-, fluva- and simvastatin were between 0.02 and 0.1 µM whereas it reached 50 µM for pravastatin. The IC_{20} of atorva-, fluva- and simvastatin were in the low micromolar range, fluvastatin

being the most potent in inhibiting HAEC proliferation (0.07 µM), followed by simvastatin (0.3 µM) and atorvastatin (0.4 µM). In contrast pravastatin exhibited a limited anti-proliferative activity (120 µM). For the 4 statins, the IC_{50} were 2 to 5 times higher to IC_{20}.

In all further experiments, the concentrations as reported in Table 3 were used to treat HAEC and alamarBlue® assays were performed in parallel to ensure proper drug effects of the IC_{50}, IC_{20} and SD on proliferation.

2. All statins exhibit an effective anti-cytomegalovirus activity

To evaluate the anti-CMV activity of statins, HAEC were treated with the 3 doses of each statin 24 hours prior to HCMV infection at an MOI of 1. HCMV titers were determined at 6 dpi in the supernatants of cultures by a $TCID_{50}$ assay. Figure 9 reveals a dose dependent anti-CMV activity exerted by all four statins.

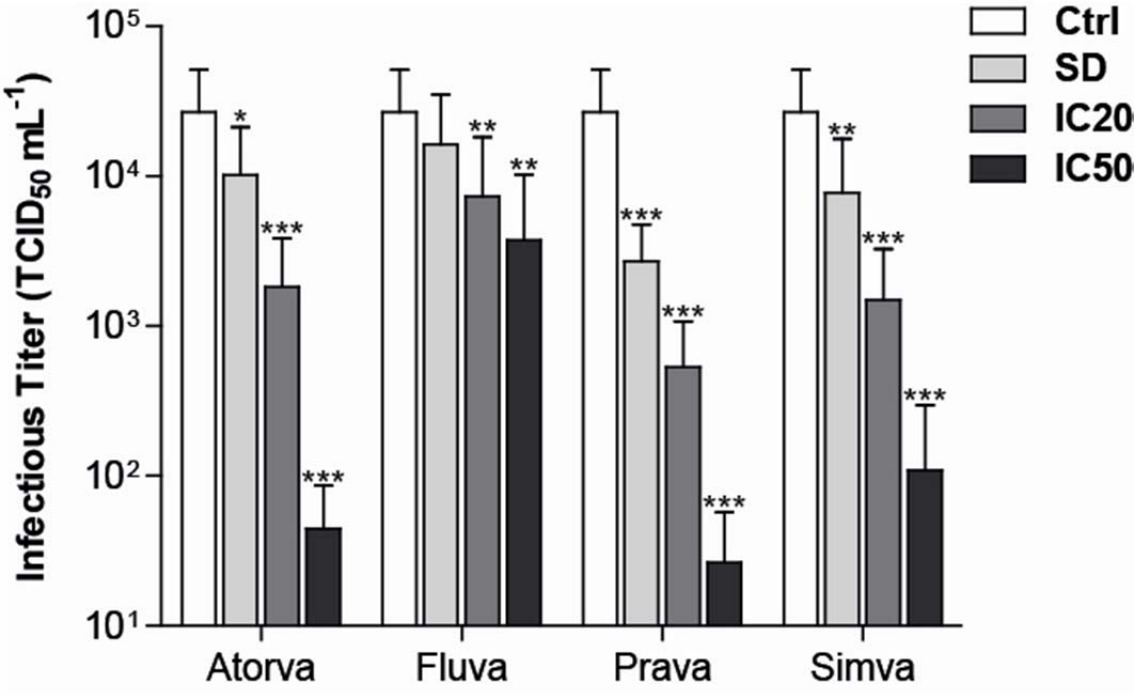

Figure 9: Statins dose-dependently inhibit HCMV infectious titers in HAEC.

HCMV infectious titers were determined in supernatant of cultures at 6 dpi by a $TCID_{50}$ assay. Results are expressed in $TCID_{50}$ mL^{-1} as mean values ± SD of five independent experiments.

At the SD, atorva- and simvastatin significantly reduced the titers by 0.4 and 0.5 log respectively; pravastatin decreased the titer by 1 log whereas fluvastatin had no effect. At the IC_{20} and the IC_{50}, all statins significantly impacted HCMV titers with fluvastatin

exhibiting the most limited effect. At the IC_{50}, HCMV titer reduction reached 2.4 log for simvastatin, 2.8 log for atorvastatin and 3 log for pravastatin.

Table 3: *In vitro* anti-proliferative and anti-CMV activity of statins on HAEC.

Cell type	Drug	Anti-proliferative activity[a] (µM)			Anti-CMV activity[b] (µM)	
		SD	IC_{20}	IC_{50}	EC_{50}	EC_{90}
HAEC	Atorvastatin	0.01	0.5	2	0.03 ± 0.05	0.17 ± 0.10
	Fluvastatin	0.01	0.1	0.3	0.03 ± 0.03	0.31 ± 0.18
	Pravastatin	50	100	400	26 ± 8	64 ± 20
	Simvastatin	0.01	0.5	1	0.003 ± 0.006	0.1 ± 0.08

[a] Concentrations responsible for 50% (IC_{50}), 20% (IC_{20}) or no cell growth inhibition (SD or sub-inhibitory dose) over a 72-hour period of time, as compared with untreated control cell growth.

[b] EC_{50} and EC_{90} values determined by $TCID_{50}$ assay. Nonlinear regression analysis was performed, and the resulting graphs were used to calculate the respective values. Results are expressed as means ± standard deviations of at least 3 independent experiments.

To compare the potential of the four statins to inhibit HCMV replication, independently of their anti-proliferative activity, we determined the effective concentration 50 % (EC_{50}) and 90 % (EC_{90}), being the concentration of product at which virus replication is inhibited by 50 and 90 percent. EC_{50} and EC_{90} were found to be within the concentration range of the SD and IC_{20} used (Table 3). Simvastatin exhibited the highest inhibitory potency (EC_{50}~3 nM) as compare to atorva- and fluvastin (EC_{50}~30 nM), although they all displayed a similar EC_{90} between 0.1 µM and 0.3 µM. In contrast, the inhibitory potency of pravastatin was drastically lower (EC_{50}~26 µM).

3. Increasing multiplicity of infection do not affect the anti-cytomegalovirus activity of statins

Liu et *al.* demonstrated that the reduction of poliovirus infection, resulting from a disruption of cholesterol-rich domains by methyl-β-cyclodextin (MβCD), was reversed by an increasing MOI [150].

Similarly, we evaluated the MOI dependency of the anti-CMV activity of statins. Atorva- and simvastatin, resulting in a similar impact on HCMV titers than pravastatin but with

much lower concentrations, were both used to treat HAEC with the IC_{50} before the inoculation with MOI ranging from 0.03 to 10 (Figure 10). The infectious titers measured in supernatants of control cultures at 6 dpi increased from 2 log $TCID_{50}$ mL^{-1} at an MOI of 0.03, up to 6 log $TCID_{50}$ mL^{-1} at an MOI of 10. No infectious progenies were detected in supernatants of cultures treated with statins and infected at an MOI ≤ 0.3, as compared to untreated cultures. Consistently, at an MOI ≥ 1, both statins exhibited similar effects and stably reduced HCMV titers by almost 3 log as compared to control cultures), suggesting that statin potency is sufficient to encounter with high-MOI infections.

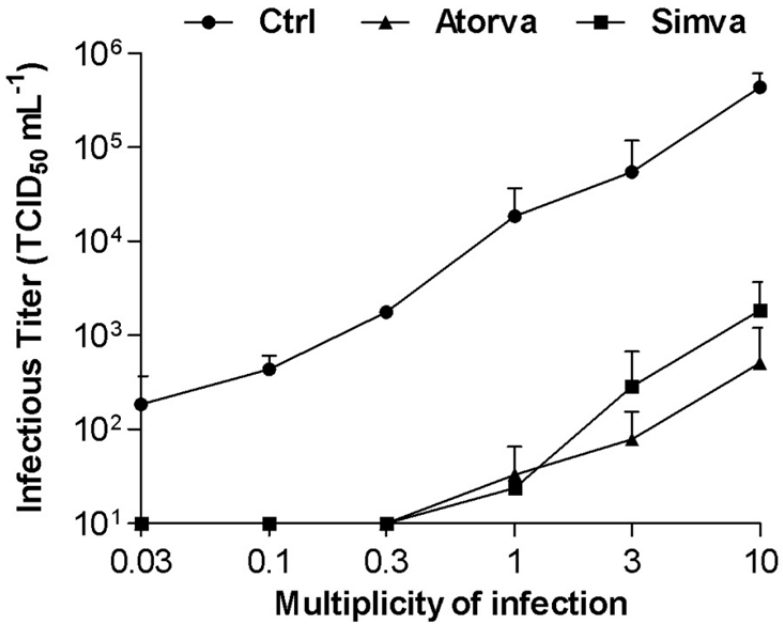

Figure 10: Influence of the multiplicity of infection on the anti-cytomegalovirus activity of statins in HAEC.

Statin-treated cultures were inoculated with HCMV at MOI ranging from 0.03 to 10. HCMV infectious titers, determined in supernatant of cultures at 6 dpi, are expressed in $TCID_{50}$ mL^{-1}. One representative experiment out of two is shown.

Although the four statins we tested differ in their chemical structures, their ability to lower cholesterol *in vivo* [130] or to inhibit cell proliferation *in vitro*, we confirmed the anti-CMV activity of fluvastatin even at lower doses than previously demonstrated [163] and that this effect is not limited to a specific statin. Furthermore, all lipophilic statins we tested manifest a potent anti-CMV activity using doses in a similar micromolar range. On the other hand, although pravastatin displayed the strongest ability to reduced HCMV infectious titers, the *in vitro* concentrations to achieve such anti-CMV effect might limit the existence of a similar activity *in vivo.*

II. Cellular and molecular mechanisms of the anti-cytomegalovirus activity of statins in endothelial cells

Endothelial cells are assumed to play a central role in the haematogeneous dissemination of HCMV through the productive infection of the vascular endothelium *in vivo,* with virus being very likely to spread from EC by release into the blood stream, contact with adjacent cells or transiently adhering leukocytes [178].

In the meantime, the protective barrier formed by vascular EC between circulating blood and the rest of the blood vessel wall is directly exposed to statins via the blood circulation and was shown to be one of the key mediators of their pleiotropic effects [134]. *In vitro*, several cellular functions of EC were shown to be modulated by statin treatment. Atorva-, fluva- and simvastatin have pro-apoptotic effect by down-regulating Bcl-2 expression [179]. Simvastatin disorganizes the actin cytoskeleton of activated EC [180]. Moreover, both atorva- and simvastatin downregulate the activation of pro-inflammatory transcription factor NF-κB [181]. All these cellular functions, among others, are required for the HCMV entry and replication and could thus be involved in the mechanism of the anti-CMV activity of statins.

To identify the mechanism of the anti-CMV activity of statins, we looked for effects of statins on EC phenotypes. In a second time, we investigated in detail the different steps of the HCMV replication cycle.

1. Effects of statin treatment on cell phenotypes

1.1 Statin treatment does not result in cellular cholesterol depletion

In fibroblasts, disruption of lipid rafts by depletion of cellular cholesterol inhibits both HCMV entry and IFN responses [182]. The first and main role of statins *in vivo* is to lower blood cholesterol levels. *In vitro*, pravastatin treatment was shown to result in a dose-dependent inhibition of macrophage cholesterol [183].

To assess whether statin treatment resulted into a disruption of the cellular cholesterol in HAEC over the period of infection, we determined the cholesterol content into the cell extract of cultures treated with statins, using the Amplex® Red cholesterol assay. A

cholesterol-protein ratio was determined for all conditions to exclude any variability due to the cellular concentration. A 1-hour treatment with MβCD (10mM), a known cholesterol depleting reagent, was used as a positive control for each time point.

Over a period of 7 days of treatment, no significant and reproducible modulation of the cellular levels of cholesterol was observed between statin-treated and untreated cultures up to 4 days. At 5 and 6 days post treatment, an increase of the cholesterol level could be observed in some of the statin-treated cultures. Nevertheless, these variations were inconsistent over repeated experiments and the co-treatment with mevalonate (MVA) never counteracted theses variations, suggesting an unspecific effect (data not shown).

No significant modulation of the cellular cholesterol level was observed in HAEC following treatment with statin that could be correlated with an inhibition of HCMV entry after 1 day of treatment or an inhibition of the virus egress after several days of treatment.

1.2 Statins modulate HCMV entry receptor expression

HCMV use several trans-membrane protein receptors to enter into cells, including EGFRand PDGFRα or co-receptors such as αvβ3 integrin or β1 integrin subunit. [14-16, 184]. Statins were shown to modulate their expressions. Atorvastatin increases the cell surface expression of α2β1 integrin on vascular smooth muscle cells, and lovastatin to downregulate EGFR expression [185, 186].

We therefore analyzed HAEC for their relative cell surface expression of HCMV entry receptors and co-receptors after a 24-hour treatment with the statins' IC_{50}. Surface expression of EGFR, PDGFRα and β1 integrins was quantified by flow cytometry. High level of β1 integrins and low levels of PDGFRα were detected on non-treated cultures but no EGFR expression (Figure 11). All statins increase the cell surface expression of β1 integrins by 27% up to 65% with atorva- and pravastatin, respectively. In comparison, only prava- and simvastatin did increased PDGFRα cell surface expression but only in a very limited manner.

Therefore, the modulation of HCMV entry receptors we observed at the time of infection rather suggests a possible increase than an inhibition of HCMV entry that could explain the anti-CMV activity of statins.

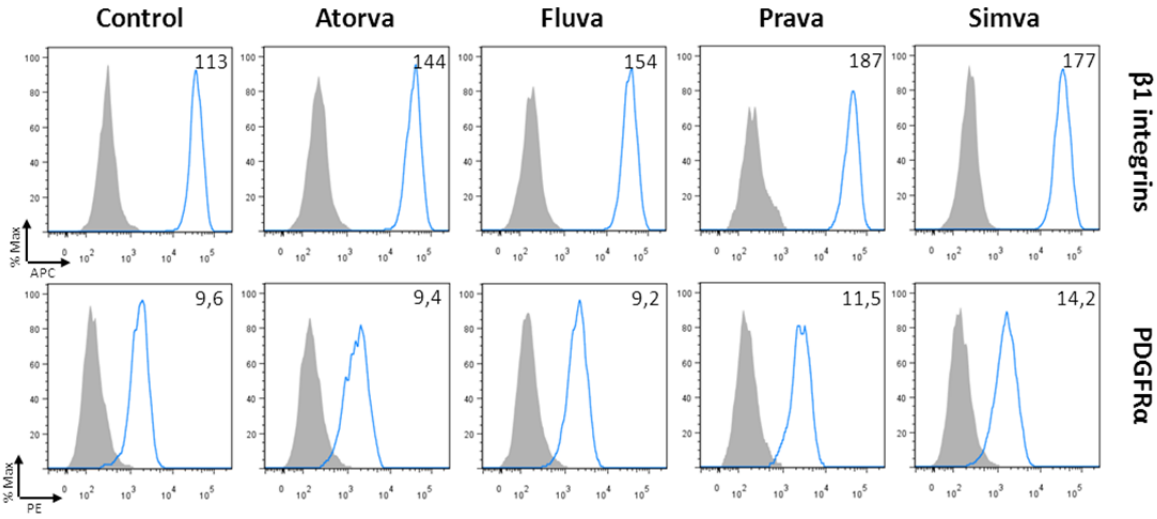

Figure 11: Effect of a 24-hour statin treatment on the cell surface expression of HCMV entry receptors on HAEC.

Histograms overlays of anti-entry receptor staining (blue) versus isotype control (grey), median fluorescence intensity ratio are indicated.

1.3 Statins do not induce a cell cycle arrest in EC

A pilot study showed that murine 3T3 cells maintained in the G1 phase permit HCMV replication [187]. Moreover, inhibition of cyclin-dependent kinase 2 (Cdk2) activity, which regulates cell cycle progression in G1 and S phase activity blocks HCMV replication, indicating that activation of cellular Cdk2 is necessary for HCMV replication [188]. Interestingly, cell cycle analysis of vascular EC reveals that 24-hour exposure to fluvastatin prevents cells from leaving G1 [189]. Simvastatin also causes a G1 arrest in different types of human melanoma cell lines [190]. Mevastatin inhibits cdk2 activity in PC3 cells through the inhibition of Thr-160 phosphorylation of cdk2 [191].

We thus decided to examine the effect of all four statins on the cell cycle progression of HAEC. Cells, seeded in similar conditions as for infection experiments, were treated with the statin IC_{50}, fixed after 1 or 4 days of treatment, stained with propidium iodide and analyzed by flow cytometry. DMSO and nocodazole were used as a G1 and G2 arrest control, respectively. After 1 day of treatment, the percentage of cells determined for each phase of the cell cycle revealed a large majority of cells in G1 in the untreated cultures (80%) and only a small fraction in the G2/M phase (10%). While DMSO increased the fraction of cells in G1 (84%) and nocodazole treatment resulted in a large shift of cells in the G2/M phase (30%), none of the statins were able to significantly modulate the cell cycle progression (Figure 12). Similar results were observed after 4

days of treatment (data not shown). In another set of experiments, although lower cell-seeding concentration were used to delay the confluency of cells and therefore a G1 arrest, no significant modulation of the cell cycle progression was observed with statin treatment in our hands.

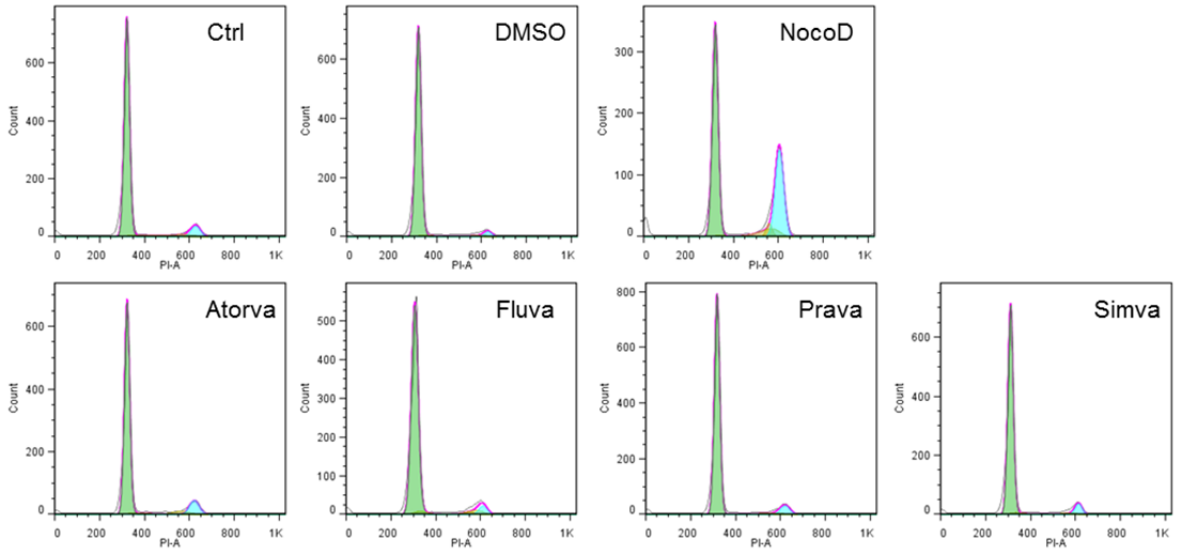

Figure 12: Effect of a 24-hour statin treatment on the cell cycle progression of HAEC.

Cell cycle analysis of cultures treated for 24 hours with DMSO, nocodazole or the statin IC_{50}. Histograms overlays of cells in G1 (green) and G2/M (blue) phases are indicated.

Using similar conditions for all our experiments, it is thus unlikely that statins exert their anti-CMV activity through an effect on the cell cycle progression since the majority of the cells already are in the G1 phase required for HCMV replication.

1.4 Statins do not stimulate the type I interferon pathway

Type I IFN is one of the most important effector cytokine inhibiting viral replication by inducing host genes expression [192, 193]. In vitro, pre-treatment of human fibroblasts with IFN-α or IFN-β was shown to inhibit HCMV plaque formation [194]. Moreover, disruption of lipid rafts by cholesterol depletion using MβCD interferes with the induction of the IFN response to HCMV infection [182]. We wondered whether the reduced HCMV titers observed upon statin treatment might result from an activation of the type I IFN pathway by statins.

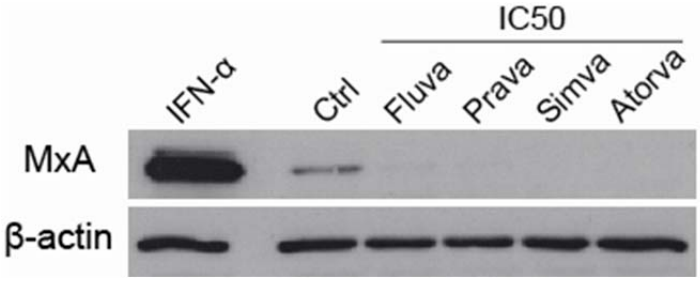

Figure 13: MxA expression in statin-treated cultures of HAEC.

Western blot analysis of the myxovirus protein A expression in cultures treated for 24 hours with the statin IC_{50} or with 1000 unit/mL IFN- α. β-actin was used as a loading control. One representative experiment out of three is shown.

To this end, we analyzed expression of the myxovirus protein A (MxA), an IFN-inducible antiviral effector, after a 24-hour treatment either with the statin IC_{50} or with 1,000 unit/mL IFN-α used as positive control (Figure 13). Compared to untreated cultures, HAEC treated with IFN-α exhibited a markedly increased expression of MxA. Inversely, MxA expression was not affected by statin treatment. Thus, statins do not exert their anti-CMV activity by targeting the pathway of the immediate host response to virus infection.

2. Effects of statins treatment on HCMV replication cycle

In the absence of any identifiable cellular function specifically affected by statin treatment that could explain the mechanism of the anti-CMV activity of statins, we next investigated potential targets within the HCMV replication cycle.

2.1 Statins do not interfere with HCMV entry

To determine if statin prevent viral attachment and/or HCMV entry into the cells, we quantified the amount of viral DNA present in cell extracts of statin treated cultures shortly after the start of the infection 2 hpi (Figure 14A). No differences in the number of viral DNA copies were observed between control and statin-treated cultures.

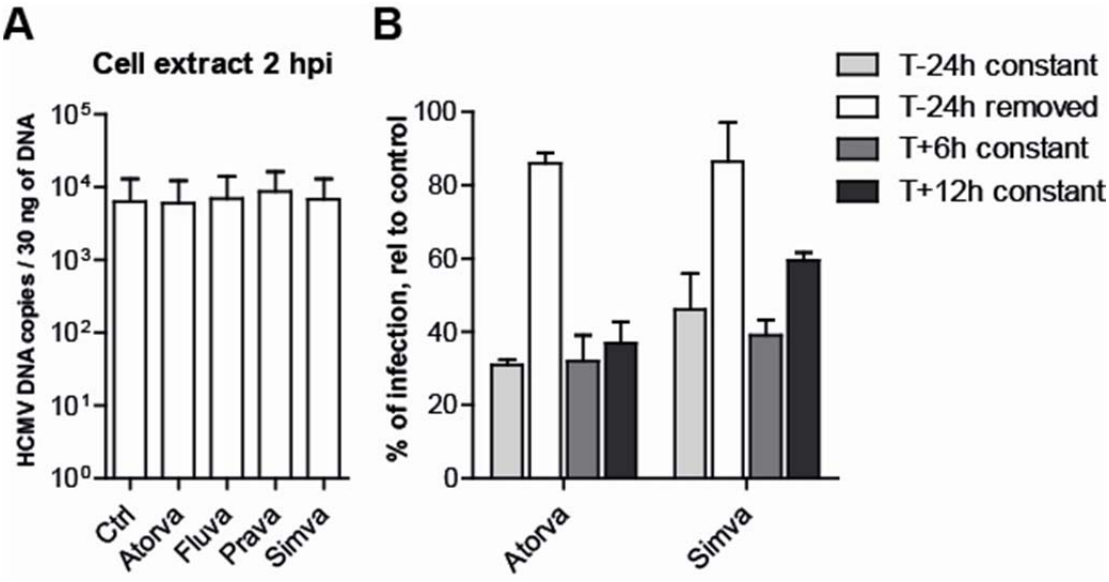

Figure 14: Statins do not interfere with HCMV entry in HAEC.

(A) HCMV DNA copies quantified by real time-PCR in the cell extract (30 ng of total DNA) of HAEC cultures at 2 hpi. One representative experiment out of two is shown.
(B) HCMV infectious titers determined at 6 dpi by a $TCID_{50}$ assay in supernatant of cultures. Statin IC_{50} were added i) 24 hours prior infection and kept until the end of the experiment (T-24h constant), ii) 24 hours prior to infection and definitively remove at the time of infection (T-24h removed), iii) 6 hours (T+6h constant) or 12 hours (T+12h constant) after HCMV infection and kept until the end of the experiment. Results are expressed in relative level of infection compared to untreated cultures, and bars represent mean values ± SD of two independent experiments.

In addition, we explored the effects of different times of drug treatment relative to virus exposure (Figure 14B). Statins were added at different time prior- or post-HCMV infection and were co-incubated for the duration of the culture or removed at the time of infection. At 6 dpi, supernatants were subjected to $TCID_{50}$ assay to determine HCMV titers. When HAEC were pre-incubated for 24 hours with statins followed by removal at the time of infection, titers almost recovered to the level of drug-free cultures (86% for both atorva- and simvastatin). Interestingly, no difference in the anti-CMV activity of statins was observed between statin treatment starting from 24 hours prior HCMV infection or 12 hours after HCMV infection. These data suggest that statins interfere with HCMV replication at a step downstream from HCMV entry.

2.2 Statins do not directly reduce virion infectivity but reduce viral DNA production

We next assessed if the observed reduction in HCMV titers was due to a decrease in the number of virions produced or the result of an alteration of their infectivity or stability. The amount of viral DNA at 5 dpi was quantified by real time PCR in the supernatants

and cell extracts of HAEC cultures treated with the IC_{50} of the respective statins (Figure 15 A&B). Compare to untreated cultures, treatment with all statins led to a marked reduction in the number of viral DNA copies in the supernatants, although limited with fluvastatin. Meanwhile, the intracellular number of copies was equally reduced, except for fluvastatin-treated cultures (Figure 15B). These data suggest a blockade of HCMV replication, occurring after HCMV entry into the cells and before the virion exocytosis, resulting in a reduced number of infectious virions released rather than a reduction of virion infectious potential.

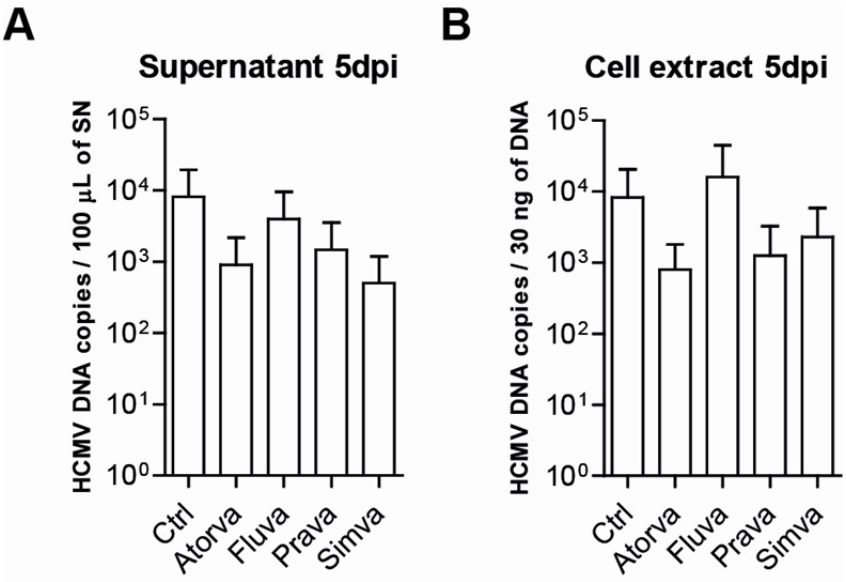

Figure 15: Statins do not interfere with viral DNA replication in HAEC.

HCMV DNA copies were quantified at 5 dpi by real time-PCR in (A) 100 µL of supernatant (SN) and (B) in the cell extract (30 ng of total DNA) of HAEC cultures. One representative experiment out of three is shown.

2.3 Statins prevent viral antigen expression

To identify the stage of viral replication which is compromised by statin treatment, time-course relative expression of the IE, E and L antigens was analyzed from 1 to 6 dpi by western blotting (Figure 16). As expected, in control cultures IE antigens were observed from 1 dpi onwards and continuously accumulated up to 6 dpi, while both E and L antigens were detected from 3 dpi onwards. Noteworthy, in statin-treated cultures, only low amounts of IE antigens were detected at 1 dpi and no further accumulation was noticed after 2 dpi. Moreover, neither E nor L antigens were detected up to 6 dpi in cultures treated with atorva-, prava- or simvastatin.

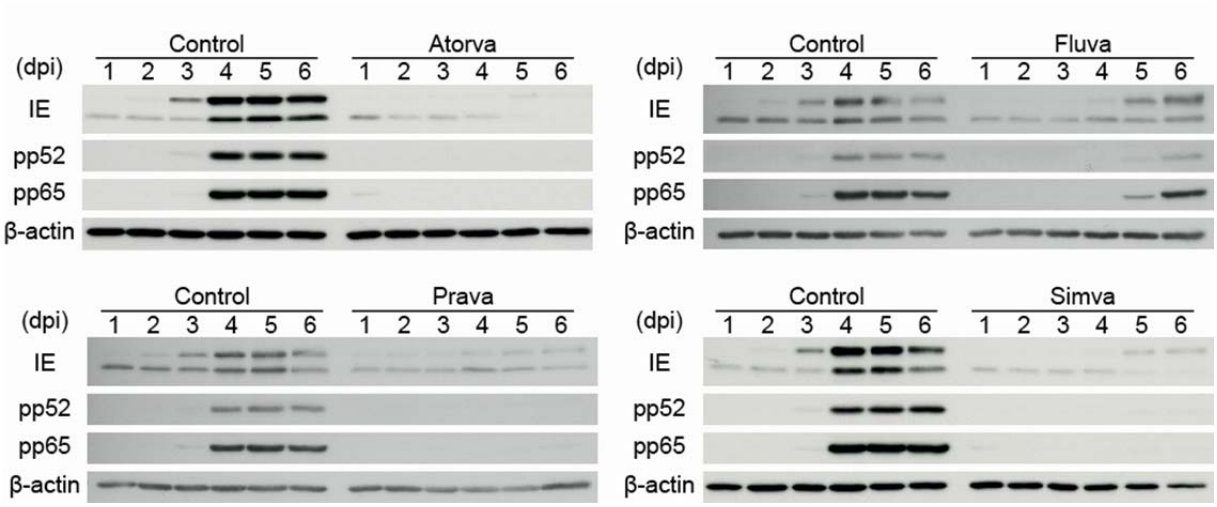

Figure 16: Statins affects the expression of the three families of viral antigens in HAEC.

Western blot analysis of viral IE, E (pp52) and L (pp65) antigen expression between 1 and 6 dpi in cultures treated with the statin IC_{50}. β-actin was used as a loading control. One representative experiment out of three is shown.

In order to exclude a specific inhibition of some antigens, we evaluated the effect of statins on others viral antigens as well. The expression of MCP, another late antigen was also dramatically inhibited in cultures treated with the atorva-, prava- and simvastatin IC_{20} at 6 dpi (Figure 17).

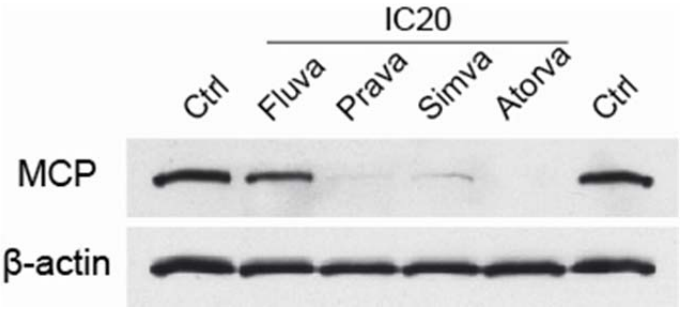

Figure 17: MCP expression in statin-treated cultures of HAEC.

Western blot analysis of the late Major Capsid Protein (MCP) expression at 6 dpi in cultures treated with the statin IC_{20}. β-actin was used as a loading control. One representative experiment out of three is shown.

Consistent with its limited effect on HCMV titers, fluvastatin treatment only moderately impacted the expression of the different viral antigens as compared to the three other statins. Though IE expression was reduced when compared to untreated control culture, accumulation of IE antigens reoccurred after 4 dpi (Figure 16). Consistently, both E and L antigens increased at 5 dpi but the level of expression remained lower than their respective controls and MCP expression was only slightly reduced at 6 dpi (Figure 17).

This suggests a delay of 3 days in the initiation of the replication cycle induced by fluvastatin while other statins achieved a stable inhibition over the whole period of infection. This may also explain the previously observed high number of viral DNA copies in the cell extract but not in the supernatant of fluvastatin-treated cultures at 5 dpi (Figure 15).

Overall, these data show that atorva-, prava- and simvastatin halted the accumulation of IE antigens and abrogated post-IE expression of viral gene products (early, early-late and late). The limited impact of fluvastatin on virus titers coincided with a moderate delay of the accumulation of IE, E and L gene products.

2.4 Statins mainly target late antigen expression

Since the 3 families of antigens are sequentially expressed and IE antigens acted as transcriptional activators for the E genes, we wondered whether the abrogation of post-IE viral gene products (early, early-late and late) expression by statins resulted from the inhibition of IE expression or if they were also specifically inhibited.

To do so, HAEC cultures were treated with the statin IC_{50} either 24 hours before the inoculation with HCMV (as for previous experiments) or at various times after the inoculation ranging from 6 to 36 hours post infection (hpi) (Figure 18 A-C). Interestingly, the reduction of HCMV titer obtained with a pre-infection treatment (T-24h) was maintained when treatment was delayed up to 18 hpi with simvastatin and 24 hpi with atorvastatin. Not only does that confirm the statin do not interfere with HCMV entry, but it suggest that statins do not only affect IE antigens expression.

Indeed, the expression of IE antigens at 3dpi disclosed that only cultures treated with atorva- or simvastatin prior to infection (T-24h) exhibit a notable reduced amount of IE antigens (Figure 18 B). In contrast, the inhibition of pp65 expression at 6 dpi was maintained in cultures treated as late as 24 hpi for atorvastatin but only up to 6 hpi for simvastatin, treatment at 18 and 24 hpi resulting in a partial inhibition only (Figure 18C).

Overall, these data show that statins halted the accumulation of post-IE antigens (early, early-late and late), independently of the inhibition of IE antigens.

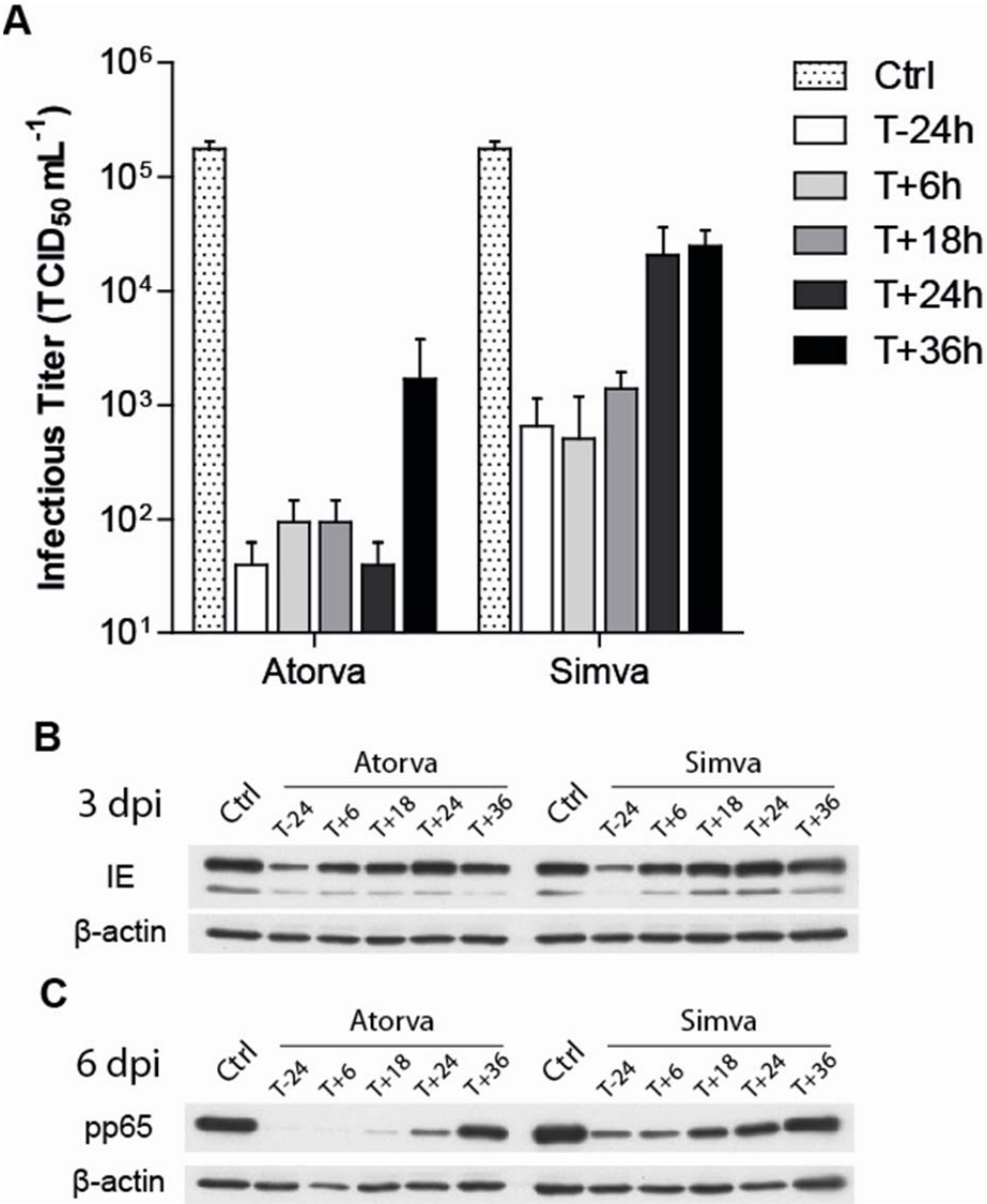

Figure 18: The anti-CMV activity of statins in HAEC persists up to a 24 hpi delay of treatment.

HAEC cultures were treated with the atorva- or simvastatin IC_{50} at various time points from 24 hours prior to infection (T-24h) up to 36 hours post infection (T+36h). (A) HCMV infectious titers were determined at 6 dpi by a $TCID_{50}$ assay in supernatant of cultures. Results are expressed in $TCID_{50}$ mL^{-1} as mean values ± SD of two independent experiments. (B+C) Western blot analysis of viral IE antigen expression at 3 dpi (B) and L antigen at 6 dpi (C): β-actin was used as a loading control. One representative experiment out of three is shown.

2.5 Mevalonate-derived products mediate the anti-cytomegalovirus activity of statins

To get more insights into the mechanism of the anti-CMV activity of statins, metabolite rescue experiments were conducted where mevalonate (MVA), geranylgeranyl pyrophosphate (GGPP) and cholesterol (CHO) were used to counteract the statin-

mediated effects. AlamarBlue® assays were first conducted to evaluate the toxicity of each metabolite alone and to determine a subtoxic dose that did not affect cell proliferation after 72 hours of culture (data not shown). The each metabolite was added to HAEC concomitantly with the statin IC_{50} treatment and HCMV titers were quantified at 6 dpi in a $TCID_{50}$ assay. Reduction in HCMV titers mediated by statins was almost completely abolished when MVA was added to the cultures (Figure 19). Meanwhile, CHO failed to reverse the anti-CMV activity of all statins, whereas GGPP partially restored HCMV titers in fluva- and simvastatin-treated cultures.

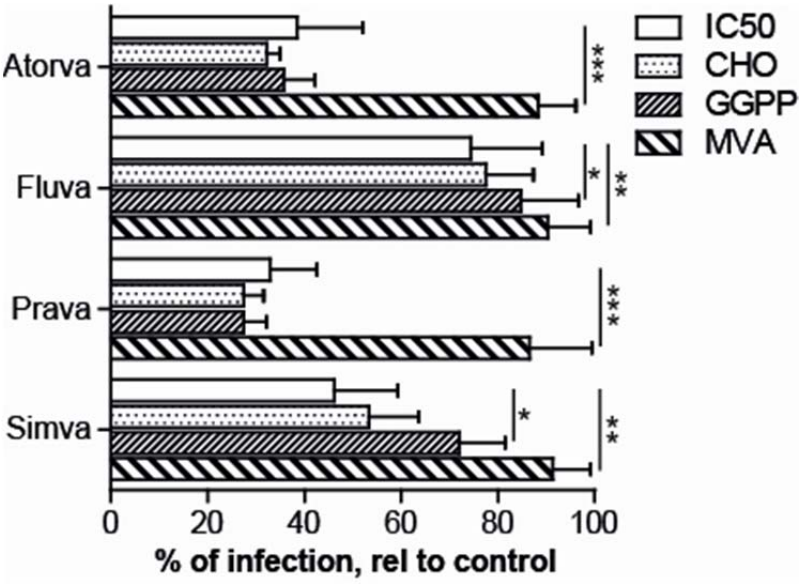

Figure 19: The anti-CMV activity of statins in HAEC is mediated by mevalonate products.

HCMV infectious titers were determined at 6 dpi by a $TCID_{50}$ assay in supernatant of cultures treated with the statins IC_{50} alone or co-treated with CHO (1 µM), GGPP (10 µM), or MVA (100 µM). Results are expressed in relative level of infection compared to untreated cultures, and bars represent mean values ± SD of at least three independent experiments.

These results demonstrate that the anti-CMV activity of statins directly results from the inhibition of the HMG-CoA reductase. The sterol branch of the mevalonate pathway seems unlikely to be involved in the anti-CMV activity of statins, but given that the inhibition of the isoprenoid intermediates play a significant role in the regulation of cell signaling processes [147], HCMV replication may be affected through the inhibition of an intracellular process.

III. Cell specificity of the anti-cytomegalovirus activity of statins

The postulated role of the cellular isoprenoids intermediates in mediating the anti-HCMV activity triggers the question of the cell specificity of the anti-CMV activity of statins. Since the mechanism seems to be cell-mediated rather than virus specific, there is a possibility that the anti-CMV activity of statins will be cell type specific.

HCMV can infect a remarkably broad range of cells within its host, including parenchymal cells and connective tissue cells of virtually any organ and various hematopoietic cell types. Skin or lung fibroblast have always been the standard cell type for isolation and propagation of HCMV from patient samples, and are still the most efficient producer cell line irrespective of the virus strain [195]. Therefore, fibroblasts were used to carry out most studies on the molecular biology and replication of HCMV. Consistent with their susceptibility *in vitro*, fibroblasts are predominantly infected during acute HCMV-infection, are a major population of cells permissive for HCMV replication and provide the platform for efficient proliferation of the virus *in vivo*[9].

Evaluation of human lung fibroblast MRC-5 may reveal some potential cell-specific differences and provide some additional understanding of the mechanism of the anti-CMV activity of statins.

1. Anti-proliferative activity of statins in fibroblasts

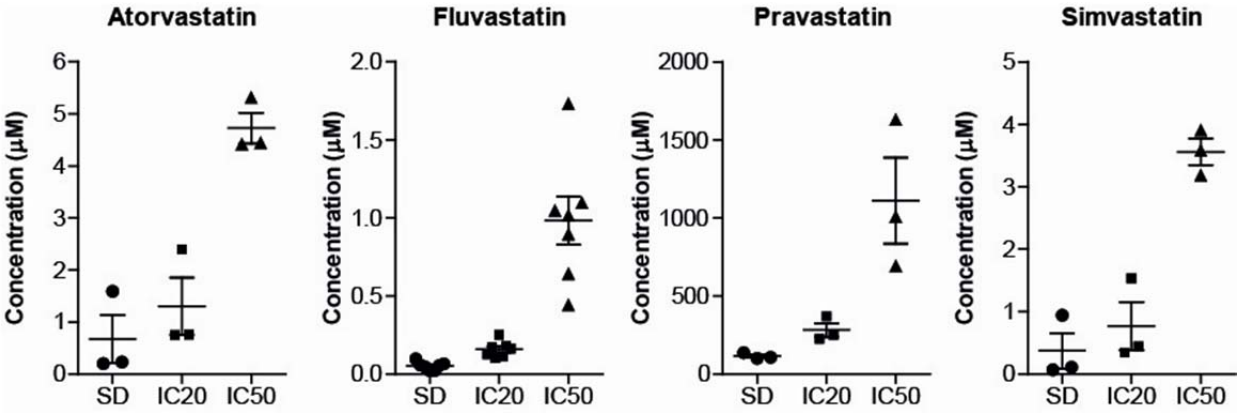

Figure 20: Anti-proliferative activity of statins in MRC-5 after a 72-hour drug exposure period.

Scatter plots of the statins concentrations responsible for 50% (IC_{50}), 20% (IC_{20}) or no cell growth inhibition (SD) as compared with untreated culture. Bar represent mean ± SEM of at least 3 experiments.

To evaluate the anti-CMV activity of the statins on the human lung fibroblast MRC-5, we first performed, as for HAEC, preliminary experiments to define the SD, IC_{20} and IC_{50} for all 4 statins using the alamarBlue® assay. Compare to EC, MRC-5 were slightly less sensitive to statins (Figure 20). The mean IC_{20} and IC_{50} values were 2- to 4- fold higher than for HAEC with the same hierarchy of the anti-proliferative activity. All lipophilic statins doses were in the micromolar range whereas pravastatin IC_{50} reached 1 mM.

2. Anti-cytomegalovirus activity of statins in fibroblasts

We next established whether statins exhibit anti-CMV activity in MRC-5 (Figure 21). Despite higher titers in supernatants of control cultures ($1x10^6$ $TCID_{50}$ mL^{-1} versus $2x10^4$ $TCID_{50}$ mL^{-1} in HAEC at an MOI of 1), a similar dose-dependent inhibition of the infectious viral particles produced in statin-treated cultures was observed. Except with pravastatin, no significant reduction of the titers were noticed with the SD. At the IC_{20}, atorva- and simvastatin lowered HCMV titers by 1 log. Pravastatin and atorvastatin achieved a 3.5 log and 2.3 log reductions of the titers at the IC_{50} while fluvastatin exhibited fairly limited anti-CMV activity on MRC-5 cultures.

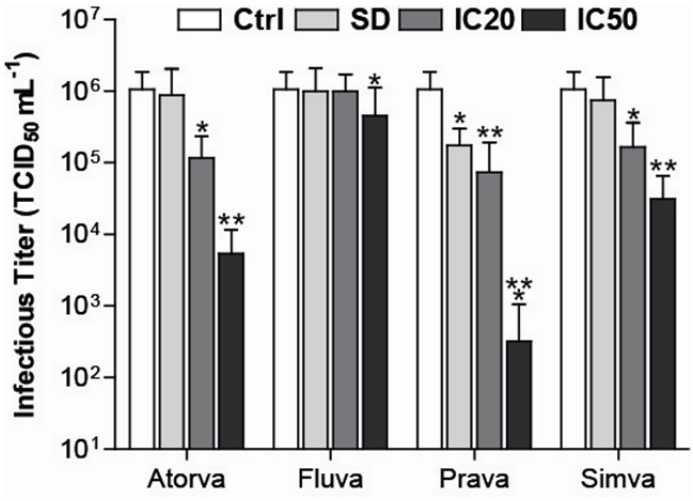

Figure 21: Statins dose-dependently inhibit HCMV infectious titers in MRC-5.

HCMV infectious titers were determined in supernatant of cultures at 6 dpi by a $TCID_{50}$ assay. Results are expressed in $TCID_{50}$ mL^{-1} as mean values ± SD of four independent experiments.

However, the determination of EC_{50} and EC_{90} revealed more discrepancy in the potency of the different statins (Table 4). Simva- and atorvastatin exhibited again the highest potency, with EC_{50} of ~0.16 µM and ~0.46 µM respectively, and fairly comparable EC_{90} (~1.5 µM and ~1.6 µM) within the range of their IC_{20}. Fluvastatin, that presented similar potency in HAEC than the 2 previous statins, was ~3 times less potent in MRC-5. Not surprisingly, pravastatin EC_{50} in MRC-5 was largely increased in comparison (~55 µM).

Table 4: *In vitro* anti-proliferative and anti-CMV activity of statins on MRC-5.

Cell type	Drug	Anti-proliferative activity[a] (µM)			Anti-CMV activity[b] (µM)	
		SD	IC_{20}	IC_{50}	EC_{50}	EC_{90}
MRC-5	Atorvastatin	0.1	1.5	5	0.46 ± 0.27	1.45 ± 0.57
	Fluvastatin	0.01	0.2	1	0.68 ± 0.37	4.08 ± 1.52
	Pravastatin	100	300	1000	55 ± 36	211 ± 116
	Simvastatin	0.1	1	3.5	0.16 ± 0.23	1.57 ± 1.04

[a] Concentrations responsible for 50% (IC_{50}), 20% (IC_{20}) or no cell growth inhibition (SD or sub-inhibitory dose) over a 72-hour period of time, as compared with untreated control cell growth.

[b] EC_{50} and EC_{90} values determined by $TCID_{50}$ assay. Nonlinear regression analysis was performed, and the resulting graphs were used to calculate the respective values. Results are expressed as means ± standard deviations of at least 3 independent experiments.

3. Effects of statin treatment on HCMV replication cycle in fibroblasts

Considering the differences in the replication rate of HCMV between fibroblasts and EC as well as the reduced anti-CMV activity of statins, we investigated the differences in the mechanism of the anti-CMV activity.

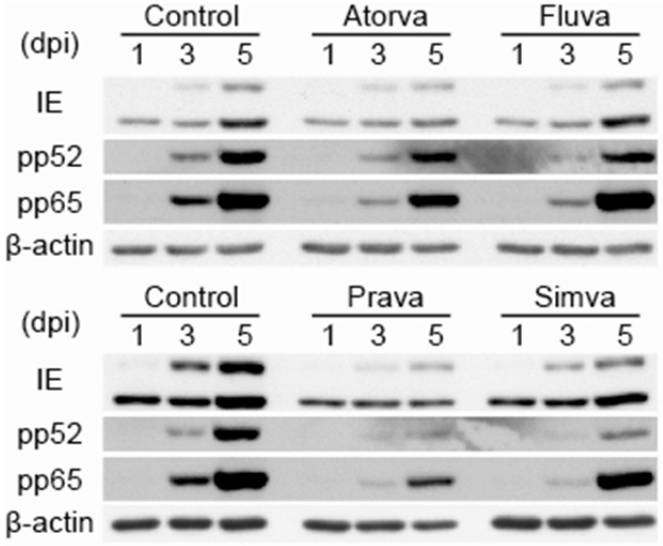

Figure 22: Statins only partially inhibit the expression of the three families of viral antigens in MRC-5.

Western blot analysis of viral IE, E (pp52) and L (pp65) antigen expression between 1 and 5 dpi in cultures treated with the statin IC_{50}. β-actin was used as a loading control. One representative experiment out of three is shown.

The time-course relative expression of the IE, E and L antigens from 1 to 5 dpi revealed slight differences compared to EC (Figure 22). In control cultures, substantial amounts of both E and L antigens were detected at 3 dpi, highlighting the increased replication rate of HCMV in MRC-5. As in HAEC statin treatment altered IE, E and L antigen expression, however, in MRC-5 accumulation of immediate-early, early and late gene products was not abolished by atorva- simva- and pravastatin but only delayed, in a similar manner than with fluvastatin in HAEC. Consistently with the reduced effect on the titre in MRC-5, fluvastatin only slightly affected the accumulation of immediate-early, early and late gene products.

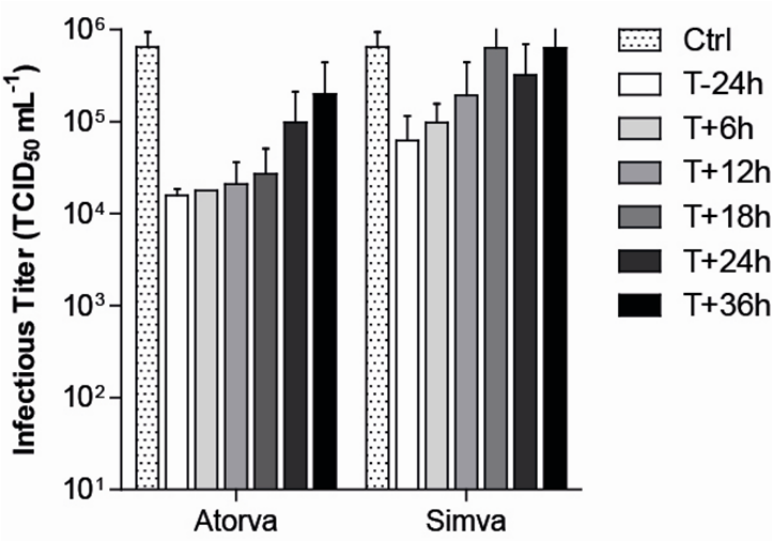

Figure 23: The anti-CMV activity of statins persist up to an 18 hpi delay of treatment in MRC-5.

MRC-5 cultures were treated with the atorva- or simvastatin IC_{50} at various time points from 24 hours prior to infection (T-24h) up to 36 hours post infection (T+36h). HCMV infectious titers were determined at 6 dpi by a $TCID_{50}$ assay in supernatant of cultures. Results are expressed in $TCID_{50}$ mL^{-1} as mean values ± SD of two independent experiments.

In delayed treatment experiments, similar results as for HAEC were obtained.. The infectious titers revealed a maintained anti-CMV activity of atorvastatin with a treatment delayed up to 18 hpi whereas simvastatin IC_{50} already partially lost its antiviral effects when added at 12 hpi (Figure 23). This earlier loss of the anti-CMV activity of statins was in accordance with the faster replication of HCMV in MRC-5 and thus the earlier expression of the E antigen. Nevertheless, this emphasizes the important role of the IE antigen inhibition in the mechanism of the anti-CMV activity in fibroblasts.

4. Isoprenoids mediate the anti-cytomegalovirus activity of statins in fibroblasts

We finally performed metabolite rescue experiments in MRC-5. Consistent with the outcome observed in HAEC, MVA almost completely counteracted the anti-CMV activity of atorva-, prava- and simvastatin, whereas CHO showed no effect (Figure 24). Interestingly, whereas in HAEC, GGPP only partly counteract the anti-CMV activity of simvastatin, it strongly abolished it for all statins, almost to a similar extent as MVA. This major difference between the two cell types could either be due to a limited uptake of GGPP in HAEC compare to MRC-5, or indicating a significant role of the down-stream isoprenoids intermediate in the mechanism of the anti-CMV activity of statins in MRC-5.

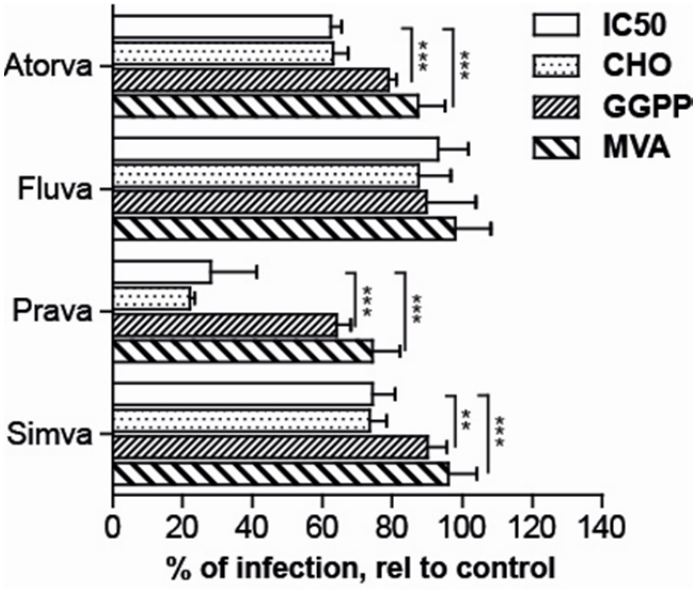

Figure 24: Isoprenoid co-treatment also counteracts the anti-CMV activity of statins in MRC-5.

HCMV infectious titers were determined at 6 dpi by a $TCID_{50}$ assay in supernatant of cultures treated with the statins IC_{50} alone or co-treated with CHO (1 µM), GGPP (10 µM), or MVA (100 µM). Results are expressed in relative level of infection compared to untreated cultures, and bars represent mean values ± SD of three independent experiments.

Altogether, the anti-CMV activity of statins did not depend on the cell type, although we could observe some differences in the replication rate of the virus in the different the cell types.

IV. Potential clinical benefits of the anti-cytomegalovirus activity of statins

1. Anti-cytomegalovirus activity and daily treatment with statin

All the results reported up to now have been achieved using one single dose of statin, used to treat cultures 24-hours before infection. However, statins are commonly prescribed as a daily treatment with "standard dose" defined to reduce LDL cholesterol by 30–45% or to prevent cardiovascular events (ie, atorvastatin 10–20 mg, fluvastatin 40–80 mg, pravastatin 40 mg and simvastatin 20–40 mg) [111]. According to clinical pharmacokinetics studies, statins are rapidly absorbed following oral administration, and reach their plasma peak concentration within 4 hours [130]. Although the elimination half-life of all statins except atorvastatin is very short (0.5–3 hours), the reported pharmacokinetic half-lives of statins do not correspond with the duration of their pharmacodynamic effect (approximately 24 hours). On the other hand, due to its long elimination half-life (15-30 hours) and the presence of detectable plasma levels of active metabolites for a prolonged period of time (>24 hours), atorvastatin can accumulate in the plasma, achieving a steady-state drug concentration after multiple doses [196, 197].

Thus, we decided to evaluate the potential of repeated doses of statins on the anti-CMV activity. HAEC and MRC-5 cultures were treated in parallel with a single dose of atorva-, fluva- and simvastatin (SD, IC_{20} or IC_{50}) or with multiple doses (SD, IC_{20}) added either every other day (EOD) or every day (Daily). Multiples IC_{50} doses were not evaluated for their anti-CMV activity because of their increased cellular toxicity. Figure 25 describes the infectious titers determined in supernatants of HAEC cultures at 6 dpi. As expected, we observed a dose-dependent inhibition of HCMV titers in cultures treated with a single dose of statin, with atorvastatin exhibiting the strongest anti-CMV activity at all doses, followed by simvastatin, and fluvastatin having limited effect. Surprisingly, repeated treatment with the SD of the three statin did not result in a reduction of HCMV titers, neither given EOD nor as daily treatment. In contrast, we observed an increased anti-CMV activity with repeated IC_{20} doses of the three statins, a daily treatment being

more efficient than EOD treatment. Interestingly, both fluva- and simvastatin daily treatment achieved greater reductions of HCMV titers as compared to a single IC_{50} dose.

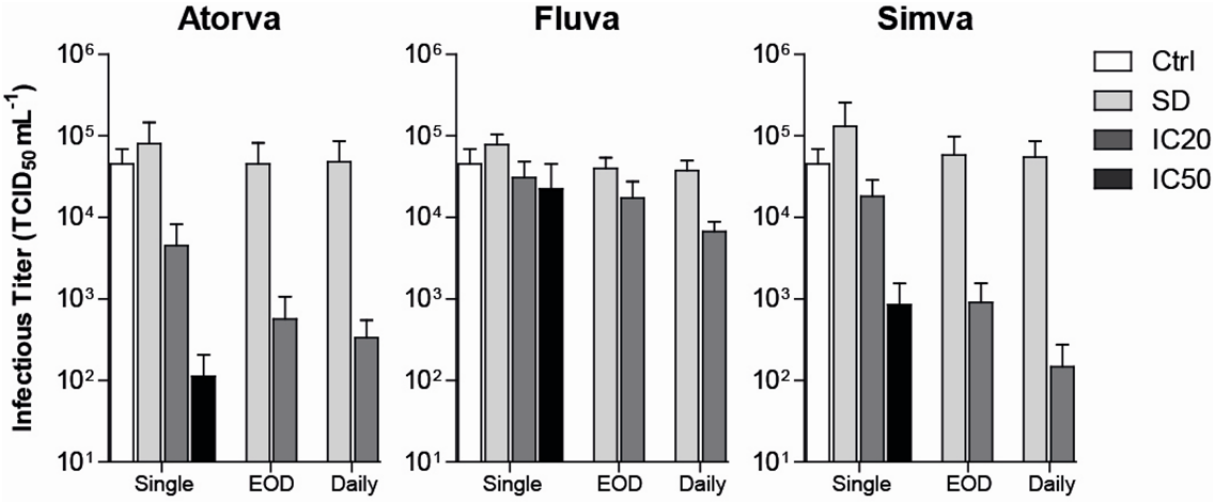

Figure 25: Additive anti-CMV activity of multiple doses of statins in HAEC.

HCMV infectious titers were determined at 6 dpi by a $TCID_{50}$ assay in supernatant of HAEC cultures treated with the indicated doses of statin either one time only (Single), every other day (EOD) or every day (Daily). Results are expressed in $TCID_{50}$ mL^{-1} as mean values ± SD of two independent experiments.

Similar results were observed on MRC-5. As for HAEC repeated treatment with the SD did not affected HCMV titers (Figure 26). With atorva- and simvastatin IC_{20}, the treatment EOD strongly improved the titer reduction obtained with a single dose, reaching similar effect than the IC_{50} with a daily treatment. However, no additivity of the anti-CMV activity of fluvastatin was observed (data not shown).

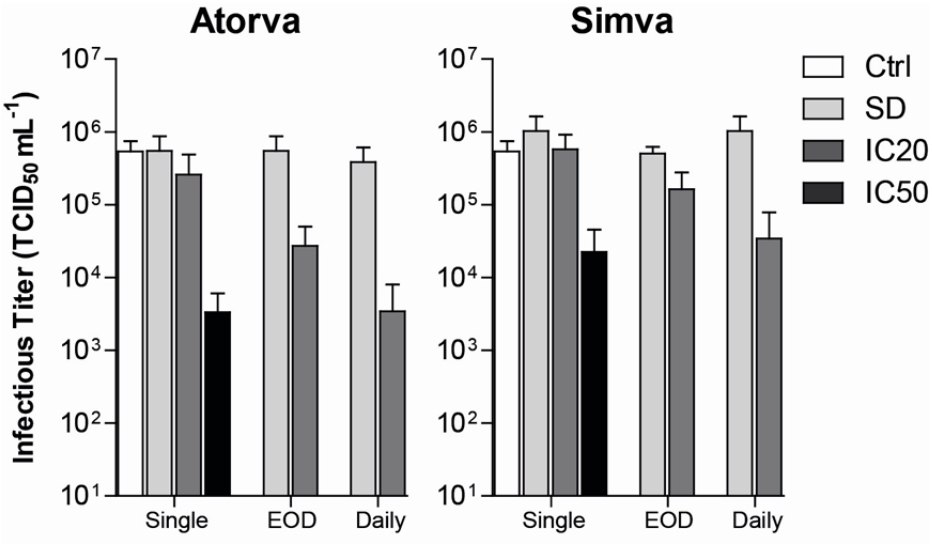

Figure 26: Multiple doses treatment with statins in MRC-5.

HCMV infectious titers were determined at 6 dpi by a $TCID_{50}$ assay in supernatant of MRC-5 cultures treated with the indicated doses of statin either one time only (Single), every other day (EOD) or every day (Daily). Results are expressed in $TCID_{50}$ mL^{-1}. One representative experiment out of three is shown.

Considering the limited concentration of statins in the blood of patients following a daily medication, it is interesting to observe an additive anti-CMV activity with small doses of statins. Nevertheless, these findings required to be confirmed *in vivo*.

2. Statins enhance the anti-cytomegalovirus activity of ganciclovir

The risk for developing HCMV resistance to antiviral drugs is greater in transplants recipients requiring long-term suppressive anti-CMV therapy. Long term management of infection may be facilitated by the administration of compounds with non-overlapping mechanisms of action as combination therapy [198]. GCV targets the activity of the viral DNA polymerase, during the early phase of infection [89]. By contrast, we showed that statins affect HCMV replication as early as the IE phase, suggesting that statins and GCV do not share the same mechanism of action. Therefore, we wondered if a combination of the two compounds would result in a synergistic anti-CMV activity.

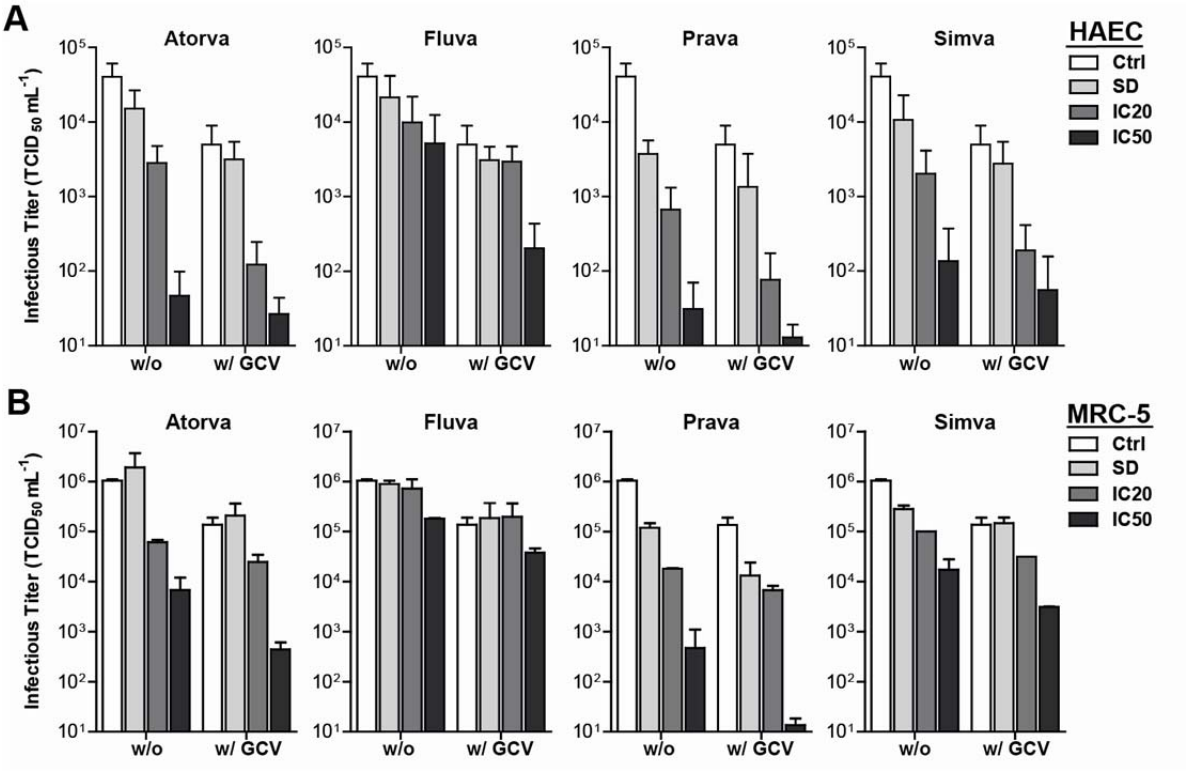

Figure 27: Statins potentiate anti-CMV activity of ganciclovir in HAEC.

HCMV infectious titers were determined at 6 dpi by a $TCID_{50}$ assay in supernatant of (A) HAEC or (B) MRC-5 cultures treated with the indicated doses of statin alone (w/o) or co-treated with ganciclovir (5 µM for HAEC, 1 µM for MRC-5) (w/ GCV). Results are expressed in $TCID_{50}$ mL^{-1} as mean values ± SD of at least two independent experiments.

Preliminary experiments were designed to evaluate the toxicity of GCV in an AlamarBlue® assay and to define a dose of GCV that would only slightly reduce (about 1 log) HCMV titers (data not shown). It is to be noted that the dose of GCV resulting in a 1 log decrease of HCMV titers in MRC-5 was 5 times lower than for HAEC (1 and 5 µM, respectively). Cultures were treated with the 3 doses of statins 24 hours prior to HCMV infection at an MOI of 1 and subsequently co-treated or not with GCV. HCMV titers were determined at 6 dpi in a $TCID_{50}$ assay.

In both cell types, statins enhanced the anti-CMV activity of GCV (Figure 27 A & B). No sign of antagonism was observed and interestingly, some combination resulted in greater effects than expected. In general, combination of GCV with the SD or the IC_{20} of every statin resulted in an addition of the anti-CMV activities. In HAEC, atorvastatin IC_{20} particularly enhanced GCV effects. While fluvastatin IC_{50} alone had only a limited anti-CMV activity (0.9 log reduction), combination with GCV further reduced the titers by 2.4 log.

These observations suggest that combination of statins with GCV resulted in interference with two distinct steps of the viral replication cycle and could at some specific doses result in a synergy of the antiviral activities.

3. Susceptibility of ganciclovir-resistant HCMV strain to statins

To confirm the previous hypothesis, we used a HCMV strain resistant to GCV, the HCMV mutant strain pp6, derived from an AIDS patient characterized with both M460I and M460V mutations of the UL97 gene [175]. Being a fibrotropic strain with a low efficiency of infection in EC, we evaluated the susceptibility of the pp6 strain in MRC-5, in parallel with the wild type fibrotropic TB40/F strain. MOI were adapted for both strains to reach similar HCMV titers in the supernatants of control cultures at 6dpi (Figure 28). As expected, increasing doses of GCV strongly affect HCMV titers of the TB40/F strain with a 3.5 log reduction with 25 µM whereas the pp6 strain replication is hardly reduced. The EC_{90} values were ~2.9 µM for TB40/F and ~140 µM for pp6, corroborating the GCV-resistance of the strain (Table 5). Note that EC_{50}s are in agreement with previously reported results [199].

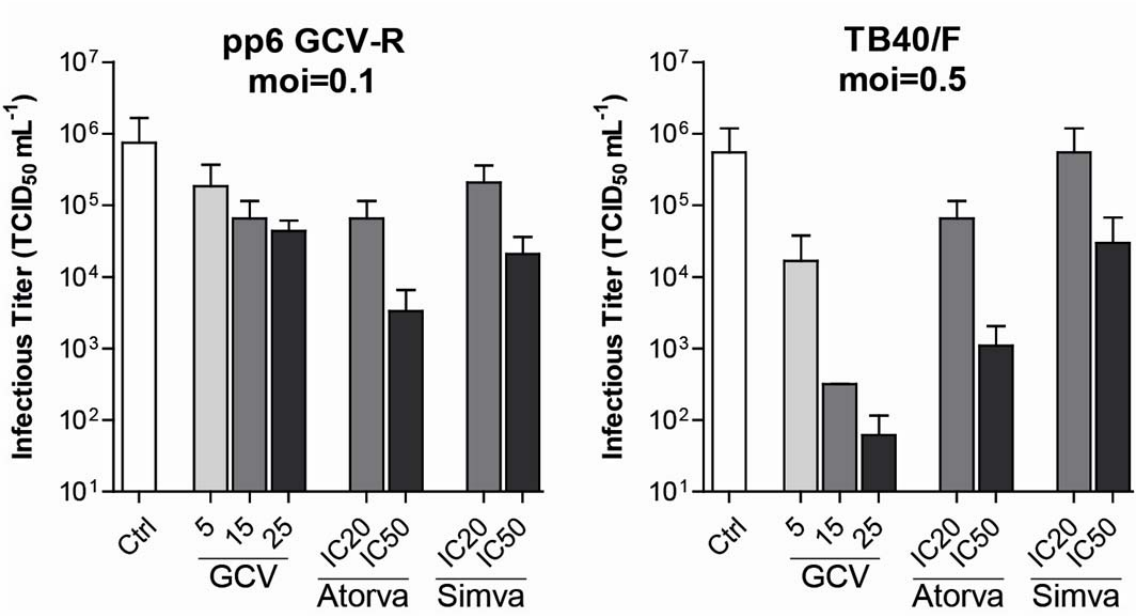

Figure 28: Statins affect both pp6 GCV-resistant and TB40/F wild-type fibrotropic strains.

HCMV infectious titers were determined at 6 dpi by a $TCID_{50}$ assay in supernatant of cultures treated with ganciclovir (5, 15 and 25 µM), atorva or simvastatin (IC_{20} or IC_{50}) and infected with the pp6 GCV-resistant strain (MOI=0.1) or with the wild type TB40/F strain (MOI=0.5). Results are expressed in $TCID_{50}$ mL^{-1} as mean values ± SD of two independent experiments.

In contrast, both strains showed similar susceptibility to statin treatment. Atorvastatin achieved a comparable drop of both pp6 and TB40/F titers. Both EC_{50} were under 1 µM and the EC_{90} equivalent to atorvastatin IC_{20} (Table 4). Simvastatin exhibit a lower potency when compare to atorvastatin, with TB40/F being slightly more susceptible than pp6, with respective EC_{90} of 1.8 µM and 2.4 µM. Both statins demonstrate an equipotent inhibitory effect on the TB40/F strain than GCV.

Table 5: HCMV resistant strain to GCV remains susceptible to statins.

	pp6		TB40/F	
	EC50	EC90	EC50	EC90
Ganciclovir	7.3	140	1.2	2.9
Atorvastatin	0.80	1.6	0.96	1.5
Simvastatin	1.7	2.4	1.2	1.8

Data are in µM.

Thus, we showed that HCMV resistance to GCV resulting from mutations of the UL97 genes does not protect the strain against the anti-CMV activity of statins.

Discussion

HCMV infection in immunocompromised host, in particular in SOT recipients, is a major cause of morbidity and mortality. Today, all available treatments for systemic HCMV infection are directly aimed at a specific viral process, the replication of the viral DNA. The characteristic latent infection often requires a long-term drug exposure of the patients during a major period of immunosuppression, which is associated with relevant side-effects. In addition, the limited bioavailability of these compounds increases the risk of ongoing viral replication resulting from insufficient antiviral drug exposure and the emergence of drug resistance. In the absence of vaccines against HCMV and considering the drawbacks limiting the use of current antiviral drugs, there is an urgent need for new therapeutic approaches for the management of acute HCMV disease.

Because viruses require the host-cell environment to replicate, one effective strategy consists in targeting a host-cell function that will affect the virus replication, with a known drug having a good safety profile. Statins are a well-tolerated and extensively studied group of drugs, primarily used as hypolipidemic agents for reducing plasma cholesterol levels and, consequently, for preventing CVD. Interestingly, favorable outcomes of statins in CVD as well as in other diseases states (for a complete review, see [147]) have been associated with several pleiotropic effects that are unrelated to their cholesterol lowering properties. Among other, antiviral properties are potentially playing an important role in mediating these effects. So far, little is known about their potential against HCMV.

In 2004, Potena et *al.* reported that fluvastatin treatment of human umbilical vein endothelial cells (HUVEC) restrained HCMV replication in vitro [163]. Although statins were shown to display cholesterol-lowering as well as cholesterol-independent class effects, their pharmacological differences (structural, biochemical and thermodynamic) raises the question of an anti-cytomegalovirus class effect. We thus decided to investigate for the *in vitro* antiviral activity of various statins against HCMV, in two relevant cell types during HCMV infection, in human aortic endothelial cells (HAEC) and in human lung fibroblasts (MRC-5).

We compared the anti-CMV activity of 4 statins, based on doses that would comparably affect the proliferative response of the treated cells. Statins revealed a similar anti-

proliferative activity in both HAEC and MRC-5, the second being slightly less sensitive (Table 3 & Table 4). Fluvastatin exhibited the stronger activity followed by simvastatin and atorvastatin, with IC_{20} under 0.5 µM and IC_{50} under 2 µM in HAEC. These concentrations are in accordance with previously published data and reveal an increased sensitivity of HAEC to statins as compared to HUVEC [179]. In contrast, pravastatin IC_{50} were about 200 times higher than the other statins in both HAEC and MRC-5. This apparent discrepancy might be explained by differences in physicochemical properties of statins. Indeed, lipophilic statins (atorva-, fluva- and simvastatin) are expected to easily and passively penetrate into *in vitro* cultured cells while the hydrophilicity of pravastatin should prevent its nonselective passive diffusion across cellular membranes [200]. However, it is generally agreed that the lipophilicity does not entirely predict the ability of statins to exert their pleiotropic effects [130, 201]. Some cells, such as primary cultured skeletal myofibres isolated from rat, express known organic anion membrane transporters, which can internalize hydrophilic statins [202]. Our findings support the notion that significant differences exist among statins with respect to their anti-proliferative activity as well as to their pleiotropic effects.

Atorva-, fluva- and simvastatin not only exhibited a similar anti-proliferative activity, but they also demonstrated an equipotent anti-CMV activity in HAEC (Table 3). We assessed EC_{50} and EC_{90} for each statin based on the reduction of HCMV infectious titers determined in the supernatants of cultures at 6 dpi. In HAEC, we found EC_{50} to be in the range of their respective SD, within the sub micromolar range for the three lipophilic statins (<30 nM). In addition, the EC_{90} appeared to be under their respective IC_{20}, with the exception of fluvastatin that reached the IC_{50} level. In contrast, higher concentrations were required to achieve similar effects in MRC-5 (Table 4). Whereas atorva- and simvastatin EC_{50} and EC_{90} did range between their respective IC_{20} and $IC_{50,}$ fluvastatin EC_{90} reached four-time its IC_{50}. Not surprisingly, pravastatin exhibited a similar low potency in term of anti-CMV activity like for anti-proliferative activity, with an EC_{50} over 50 µM in MRC-5. Altogether, simvastatin was shown to be the most effective statin to inhibit HCMV replication in both HAEC and MRC-5, with atorvastatin exhibiting almost a similar potential while fluvastatin was slightly less efficient. In contrast pravastatin displayed no effective response as assessed by the large concentrations of statin required.

We confirmed previously published data of Potena et *al.*, reporting the *in vitro* anti-CMV activity of fluvastatin treatment on HUVEC where a reduction of HCMV yield by 65% was obtained using a dose of fluvastatin of 0.1 µM while a dose of 0.2 µM reduced the yield by more than 90% [163]. In this setting, the viral yield reduction was associated with a stable reduction of IE antigen expression over a period of 14 days. We obtained a similar reduction of the viral yield after 6 dpi, with an EC_{90} of 0.3 µM in HAEC. However, the expression of the different proteins of the viral replication cycle was only moderately impacted in our hands. Inhibition of IE antigen expression was only delayed and accumulation reoccurred after 4 dpi (Figure 18). This apparent discrepancy may be attributable to differences in the treatment protocol, as in our setting, cells were treated with statins only once, 24 hours prior to infection, whereas Potena et *al.* repeatedly treated the cultures every 72 hours by adding fresh fluvastatin. Consistently, fluvastatin exhibit a shorter half-life (0.5-2.3 hours) as compared to the other lipophilic statins (atorva- and simvastatin), which could explain why only repeated treatment resulted in a similar sustained inhibition of viral protein expression achieve with a single treatment by the other statin [130].

The hypothesis of a cell specificity of the anti-CMV activity of the statins was raised, between different cell types as well as within EC from different vascular origins. EC are a highly diverse cell type according to anatomic location and tissue source. Although they share a large number of phenotypic similarities that reflect their involvement in common processes, they are widely diverse in terms of morphology, antigen expression as well as gene expression profiles that results from tissue-specific functions [203]. In particular, DNA array analyses show that micro- and macrovascular ECs vary considerably in expression levels of various molecules involved in HCMV entry, such as integrins and epidermal growth factor receptors, resulting in different susceptibility to HCMV infection [204]. However, not only did we and others observe a similar anti-CMV activity of fluvastatin in HAEC and HUVEC, but we also obtained equivalent anti-CMV activity with the 4 statins in human cardiac microvascular EC during preliminary experiments (data not shown). We could thus exclude a mechanism that would rely on a tissue-specific function.

Similarly, the cell type specificity was assessed on fibroblasts where certain differences could be observed. The ranking of statins potency was identical to the one seen in HAEC, however the concentrations required to achieve a similar anti-proliferative activity were

3-fold higher, and 10-fold higher for the anti-CMV activity. Lipophilic statins are considered to penetrate into cells by passive diffusion across cellular membranes, which should not largely differ between cell-types [200]. Thus, these disparities could be attributed to the increased metabolism of fibroblasts. Indeed, the proliferation rate of MRC-5 is twice higher than for HAEC (data not shown), while the replication rate of HCMV in fibroblasts is also clearly higher, as assessed by the viral titers determined in the supernatants of non-treated cultures at 6 dpi ($1x10^6$ $TCID_{50}$ mL^{-1} versus $2x10^4$ $TCID_{50}$ mL^{-1} in HAEC at an MOI of 1) (Figure 9 & Figure 21). Accordingly, whereas the expression of the three families of viral antigens was completely abolished in HAEC by all statins except fluvastatin during a relative time course of expression over 6 dpi (Figure 18), they were only partially inhibited in MRC-5 (Figure 22), with a similar delay of the expression as observed in fluvastatin-treated cultures of HAEC. Consistently, in delayed treatment experiments the anti-CMV activity of atorvastatin was mainly maintained when statins were added to the cultures up to 24 hpi in HAEC but only up to 18 hpi in MRC-5. However, the main difference we observed was related to the mediators of the anti-CMV activity.

The potential antiviral activity of statins has been investigated for a number of viruses and rather diverse mechanisms have been identified. In Rotavirus and Dengue virus infections, the anti-cholesterol effect explains how statins inhibit virus production via decreasing virion assembly [151, 166]. In the case of anti-HCV and anti-HIV-1 activity, statin effects seem dependent on derivatives of the mevalonate pathway, such as GGPP (Table 2) [152, 167, 168]. Our study confirms previous findings that the anti-CMV activity of statins directly resulted from the inhibition of the HMG-CoA reductase since reduction of HCMV titers and viral antigens expression were reversed by the addition of mevalonate in both cell types (Figure 19, Figure 24 & data not shown) [163]. Metabolite rescue experiments performed with cholesterol suggest that the sterol branch of the mevalonate pathway is unlikely to be involved since the co-treatment of statins with water soluble cholesterol failed to restore the anti-CMV activity in HAEC or in MRC-5. One might argue that cholesterol was not necessarily taken up by cells in culture and thus cannot counteract statins effects. However, we failed to observe any significant modulation of the cellular cholesterol level in statin-treated cultures of HAEC over a period of 7 days. Thus, even though the *de novo* synthesis of cellular cholesterol was inhibited by statin treatment, cells can most likely balance the depletion of endogenous

cholesterol via uptake from lipoproteins from the extracellular milieu through receptor-mediated mechanisms [205]. In contrast, the isoprenoid arm of the pathway might be implicated in the anti-CMV activity of statins. In HAEC, co-treatment with GGPP partially counteracted the inhibition of HCMV titers mediated by fluva- and simvastatin, but not the other statins (Figure 19). However in MRC-5, the anti-CMV activity of all statins was blocked. Consistently, a recent study performed on a model of murine CMV infection of mouse embryonic fibroblast cells showed that simvastatin exerts a dose-dependent anti-viral activity (EC_{50}=2 μM) that was shown to be dependent on GGPP inhibition but not cholesterol, in a similar series of metabolite rescue experiments [206].

So far, to our knowledge none of the HCMV proteins was reported to be prenylated, in contrast to the pseudorabies virus Us2 tegument component [207]. Even though the treatment of infected cells with lovastatin was shown to impair the Us2 protein localization by preventing its proper membrane association, this protein was not required for *in vitro* replication and uninvolved into pseudorabies virus virulence. In addition, Desplanques et *al.* demonstrated that the statin-mediated reduction of pseudorabies virus infectivity and stability of the provirus was associated with the reduction of cellular cholesterol [164]. Thus, the anti-CMV activity of statins is most likely achieved through modification of a host cell function. By interfering with several transcriptional and post-transcriptional events, mevalonate metabolites affect various biological processes. We looked for a modulation of the cell cycle progression of statin-treated HAEC (Figure 12) that might impair HCMV replication. However, in our experimental conditions a majority of HAEC was already into the G1 phase that is necessary for a proper HCMV replication [187], and no increase of the fraction of cells in G2 was observed. Isoprenoids were also shown to be involved in the energy metabolism regulation. Goto et *al.* showed that the isoprenoid FPP may serve as an endogenous agonist of PPARγ, an isoform of the PPAR subset of nuclear receptors, which binds to DNA and regulates gene expression in adipocytes [208]. These authors also demonstrated that in the presence of lovastatin, both intracellular FPP levels and PPARγ-target gene expressions were decreased. In a recent study, Rauwel et *al.* demonstrated that i) HCMV infection led to increased PPARγ activity and ii) PPARγ antagonist treatment provoked a drastic decrease in HCMV production *in vitro* [209]. In addition, by regulating the expression of IE2 mRNA PPARγ seems to play a central role in HCMV replication in a way that depends on association with its ligand. Therefore, the

anti-CMV activity observed in our hands could be the result of a drastic reduction of available FPP in the cells, preventing the FPP/ PPARγ ligand-receptor association leading to a severe reduction of viral protein production.

Potential clinical benefits of statin therapy in the management of acute CMV infection can only be achieved if clinical pharmacokinetics of statins are consistent with effective concentrations for anti-CMV activity. We determined *in vitro* EC_{50} in HAEC within the sub micromolar range for atorva-, fluva- and simvastatin (<30 nM) with a single treatment (Table 3). These concentrations are in the range of the plasma peak concentration (Cmax) reported for a 40 mg oral dose of the three lipophilic statins and thus in accordance with therapeutically relevant concentrations [130]. It is however to be noted, that the EC_{50} determined in fibroblasts can only be achieve with fluvastatin, which is the only statin reported to have a Cmax in the micromolar range. Fluvastatin could thus be potentially considered as being the more clinically relevant. Yet fluvastatin also present the shortest elimination half-life of available statins (0.5–2.3 hours compared to 15-30 hours for atorvastatin) resulting in a relatively short-lived systemic exposure. *In vitro* repeated treatment with statins significantly increased the anti-CMV activity as compare to a single dose treatment (Figure 25 & Figure 26). Consistently, a recent study performed on a model of murine infection showed that daily administration of simvastatin in mice resulted in a 1 log decrease of murine CMV viral titers in multiple organs after 4 dpi [206]. This study confirmed the *in vivo* potential of statins in control of CMV infection, however, additional studies will be necessary to evaluate the anti-CMV profiles of the different statins, alone, but also in combination therapy with conventional antiviral drugs.

Several reports have already described the ability of statin treatment to potentiate conventional antiviral therapy. *In vitro*, statins were shown to potentiate the anti-HCV activity of several HCV inhibitors [158, 169]. Delang et *al.* established that combination of mevastatin or simvastatin with IFN-α or HCV polymerase or protease inhibitors resulted in an additive antiviral activity. Moreover, the authors demonstrated that statin treatment also prevented or at least delayed the emergence of drug variants resistant to a STAT-C inhibitor. We evaluated the potential of statins in combination therapy with a low dose of GCV, the first-line treatment of HCMV infection. At most concentrations, the anti-CMV activity of statins was additive in combination with GCV and a possible potentiation was observed with certain doses (Figure 27), although additional

experiments will be required to properly assess a potential synergy of the two drugs. Nevertheless, these findings suggest a possible benefit with the use of statins therapy *in vivo,* in combination with GCV. On the contrary, maribavir, a CMV UL97 kinase inhibitor in development, was shown to antagonize the anti-CMV effect of GCV [210].

A major concern in the management of systemic HCMV infection is the emergence of drug resistance to different anti-CMV drugs and in particular to GCV. Long-term and/or repeated therapy in immunocompromised patients with severe HCMV disease is a risk factor for the emergence of mutant virus strains, not only resistant to GCV, but also cross-resistance to either or both second line agents, resulting in treatment failure [88, 89]. We demonstrated here that atorva- and simvastatin exhibits comparable anti-CMV activity than GCV in the wild-type TB40/F strain (EC_{50}~1 µM), confirming previous finding in human and murine CMV [199, 206]. Moreover, mutations of the HCMV UL97 genes (M460I and M460V) conferring GCV resistance to HCMV did not prevent the anti-viral activity of atorva- and simvastatin, EC_{50} and EC_{90} being similar in both susceptible and resistant strains (Figure 28 & Table 5). Mutations of the gene coding for the viral UL97 kinase appears in more than 80% of resistant clinical isolates from patients who receive GCV as initial therapy [88, 211]. Being consistently found in limited number of codons, UL97 resistant mutations are relatively easy to evaluate. Resistance to the UL54 *pol* gene coding for the viral DNA polymerase are less common and more likely to emerge after prolonged GCV exposure [212]. However, not only are *pol* mutations more varied and widely distributed, but also all known GCV resistance mutations in *pol* confer cross-resistance to CDV and/or FOS. It will then be of major interest to i) evaluate the sensibility of such strains to statins *in vitro* as well as *in vivo*, ii) to evaluate whether a combine statin-GCV treatment can prevent the emergence of the different kind of GCV-resistant HCMV strain.

Another multifunctional compound has recently been demonstrated as a non-specific protective agent against HCMV infection. Artesunate is a semi-synthetic derivative of the natural product artemisinin, highly effective in the treatment of severe malaria, particularly as a part of combination therapies with other antimalarial drugs. Along with a high safety and tolerability profile demonstrated in clinic, artesunate reveals a broad spectrum antiviral activity against herpesviruses and in particular against HCMV [213]. Not only artesunate does inhibit the replication of laboratory and clinical drug-resistant and drug-sensitive HCMV strains *in vitro* at therapeutically relevant doses, but the

antiviral activity of artesunate was also additive when used in combination with each one of conventional anti-HCMV drugs [214]. Artesunate is suspected to inhibit the HCMV-induced DNA binding activities of both NF-κB and SP1 transcription factors, thus interfering with critical host-cell-type and metabolism requirements in the initial step of HCMV replication. Importantly, artesunate has been successfully used for the treatment of a patient developing a drug-resistant CMV infection during preemptive antiviral therapy (with foscarnet) after stem cell transplantation [215].

With a similar profile as artesunate; a good safety profile; a potent *in vitro* anti-CMV activity alone and in combination with approved antiviral drugs; a cellular rather than a viral target limiting the risk of development of drug resistance; we believe that statins could offer a new therapeutic option for the management of acute CMV disease.

Conclusion and outlook

This work demonstrates for the first time that various statins exert a potent anti-CMV activity *in vitro* at therapeutically relevant concentrations. Statins inhibit the expression of three families of viral antigens, the immediate-early, early and late antigens, resulting in a major decrease of the production and release of infectious viral particles in endothelial cells and in fibroblasts. These effects appear to results from the specific inhibition of a host-cell functions mediated via the inhibition of the isoprenoid arm of the mevalonate pathway. Furthermore, statins demonstrated a similar anti-CMV activity in susceptible and resistant HCMV strains as well as beneficial outcome in co-treatment with ganciclovir.

These findings support further investigations on statins *in vivo*, in a murine model of CMV infection, and in a clinical setting. The evaluation of combination therapy of specific antiviral drugs with statins, as part of a cholesterol-lowering regimen or an anti-atherosclerosis therapy, might offer great benefit in the long-term management of HCMV infections in patients such as SOT transplants recipients or patients with AIDS. Combination therapy could not only increase the efficacy of current anti-CMV therapy, but also prevent the emergence of HCMV-resistant infection.

In addition, we believe that the identification of host-cell functions essential for viral replication might offer a new reservoir of target for the development of new therapeutic strategies.

Annexes

1. Bibliography

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2. Abbreviations

CDV: cidofovir
CHO: cholesterol
Cmax: plasma peak concentration
CRP: C-reactive protein
CVD: cardiovascular diseases
dpi: days post infection
ds: double-stranded
E: early
EBV: Epstein-Barr virus
EC: endothelial cells
EGFR: epidermal growth factor receptor
FOS: foscarnet
FPP: farnesyl pyrophosphate
GCV: ganciclovir
GGPP: geranylgeranyl pyrophosphate
HAART: highly active antiretroviral therapy
HC: hypercholesterolemia
HCMV: human cytomegalovirus
HCV: hepatitis C virus
HMG-CoA: 3-hydroxy-3-methylglutaryl coenzyme A
hpi: hours post infection
ICAM-1: intracellular adhesion molecule
IE immediate early
IFN: interferon
IV: intravenous
L: late
LDL: low-density lipoprotein
LFA-1: leukocyte function-associated antigen-1
MCP: major capsid protein
MCP: major capsid protein
MIEP: major immediate early enhancer-promoter
min: minute
MOI: multiplicity of infection
MVA: mevalonate
MβCD: methyl-β-cyclodextin
ORF: open reading frames
pp: phosphoprotein (ex:pp65)
SOT: solid organ transplantation.
U_L: unique long segment
U_S: unique short segment
VSMC: vascular smooth muscle cells

3. Publication

The major findings of this work have been submitted for publication in the Journal of Medical Virology as a research article.

Statins Demonstrate a Broad Anti-Cytomegalovirus Activity *in vitro* in Ganciclovir-susceptible and resistant Strains

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Running Head: Anti-CMV activity of statins

Keywords:
human cytomegalovirus (HCMV); statins; antiviral activity; endothelial cells; ganciclovir-resistance;

Abstract:

Vasculoprotective and cholesterol-lowering properties are hallmarks of statins. Recently, statins have been found to exhibit antiviral activity. Little is known about the potential of statins against human cytomegalovirus (HCMV), a risk factor in the pathogenesis of atherosclerosis. In this study, the *in vitro* anti-CMV activity of four statins (atorva-, fluva-, prava- and simvastatin) was explored in human aortic endothelial cells (HAEC) and fibroblasts. All statins dose-dependently reduced HCMV titers in both cell types. Whereas atorva-, fluva- and simvastatin showed comparable EC_{50} and EC_{90} within a low micromolar range in HAEC, pravastatin exhibited only limited effects. In metabolite rescue experiments, mevalonate almost completely abrogated the anti-CMV activity of all statins, whereas cholesterol failed to counteract the effects. Geranylgeranyl-pyrophosphate partially reversed the anti-CMV activity of most statins, suggesting an involvement of the non-sterol isoprenoid arm of the mevalonate pathway as the mode-of-action. The accumulation of immediate early viral antigens was blocked after 1 dpi onwards, and early and late antigen expression was completely abolished in HAEC. The antiviral activity of statins was comparable to ganciclovir and was retained in a ganciclovir-resistant HCMV strain. These findings provide new insight into the beneficial effects of statins, adding antiviral activity against HCMV to their list of pleïotropic properties, and support further clinical investigations on combined therapy for the management of active HCMV disease.

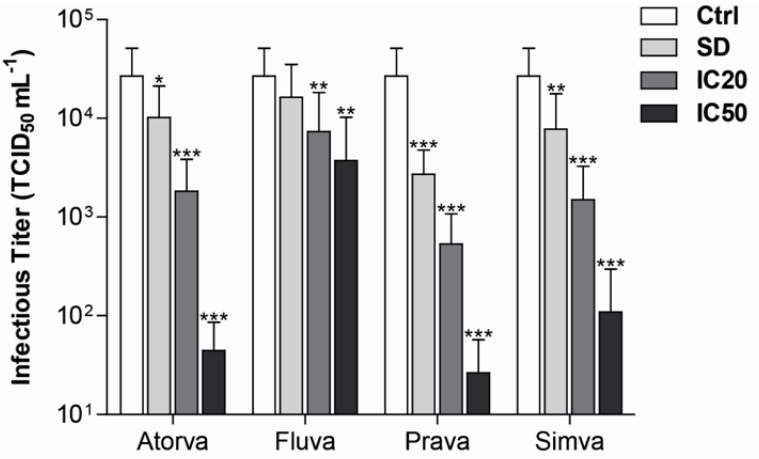

FIG 1. Statins dose-dependently inhibit HCMV infectious titers in HAEC.

HCMV infectious titers were determined at 6 dpi by a TCID50 assay in supernatants of HAEC cultures treated with the indicated doses of statins. Results are expressed in TCID50 mL-1 as mean values ± SD of five independent experiments. Comparisons were performed using analysis of variance followed by Fisher's exact test and significant differences are indicated in the graph as follows: *$p < 0.05$,**$p < 0.01$,***$p < 0.001$.

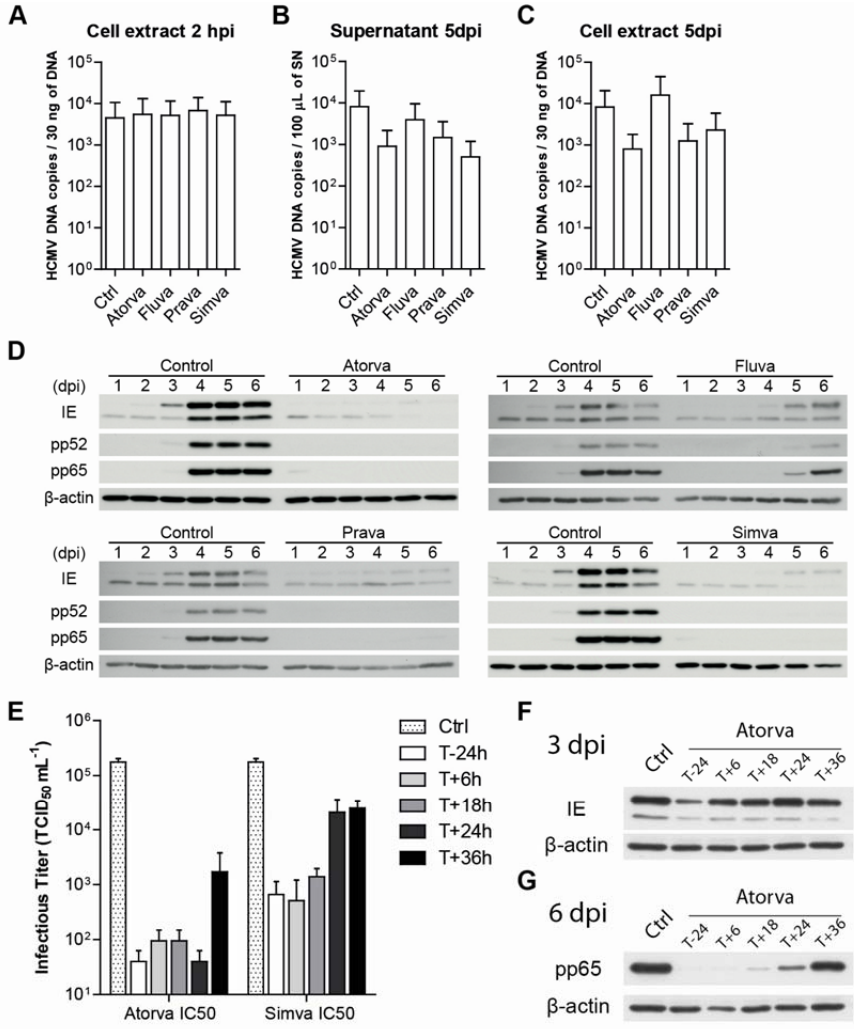

FIG 2. Statins do not interfere with HCMV entry but affects the expression of the three families of viral antigens.

(A-C) HAEC were treated with the statin IC50 and inoculated with HCMV at an MOI of 1. HCMV DNA copies were quantified by real time-PCR (A) at 2 hpi in cell extract (30 ng of total DNA) and at 5dpi (B) in 100 µL of supernatant (SN) of cultures or (B) in cell extract. One representative experiment out of three is shown. (D) Western blot analysis of viral IE, E (pp52) and L (pp65) antigen expression between 1 and 6 dpi in HAEC treated with the statin IC50. β-actin was used as a loading control. One representative experiment out of three is shown. (E-G) HAEC were treated with the atorva- or simvastatin IC50 at various time points from 24 hours prior to infection (T-24h) up to 36 hours post infection (T+36h). (E) HCMV infectious titers were determined at 6 dpi by a TCID50 assay in the supernatant of cultures. Results are expressed in TCID50 mL-1 as mean values ± SD of two independent experiments. Western blot analysis of viral IE antigen expression at 3 dpi (F) and L antigen at 6 dpi (G): β-actin was used as a loading control. One representative experiment out of three is shown

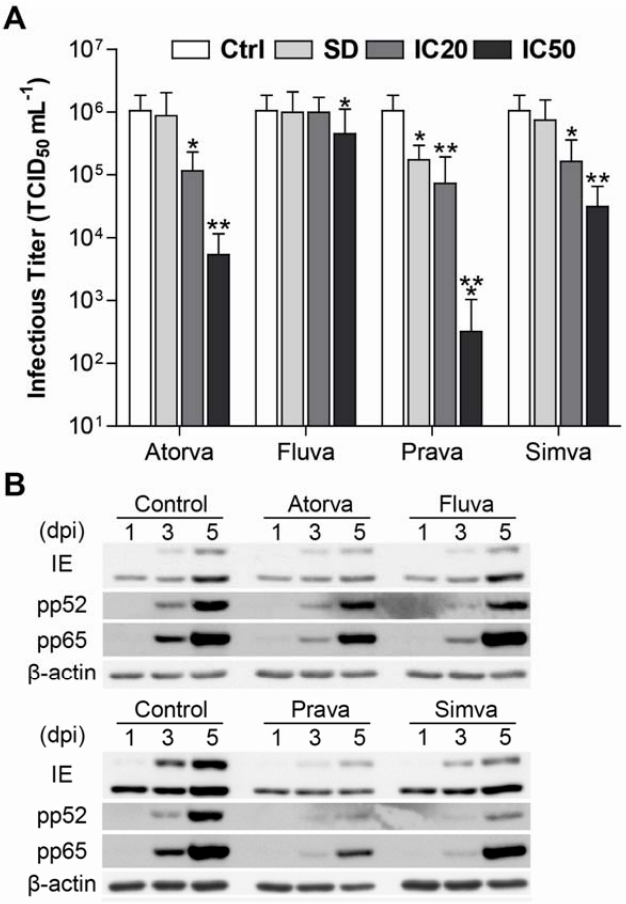

FIG 3. Anti-CMV activity of statins in fibroblasts.

(A) HCMV infectious titers were determined at 6 dpi by a TCID50 assay in supernatant of MRC-5 cultures treated with the indicated doses. Results are expressed in TCID50 mL-1 as mean values ± SD of four independent experiments. Comparisons were performed using analysis of variance followed by Fisher's exact test and significant differences are indicated in the graph as follows: *p < 0.05,**p < 0.01,***p < 0.001. (B) Western blot analysis of viral IE, E (pp52) and L (pp65) antigen expression in MRC-5 treated with the statin IC50 between 1 and 6 dpi. β-actin was used as a loading control. One representative experiment out of three is shown.

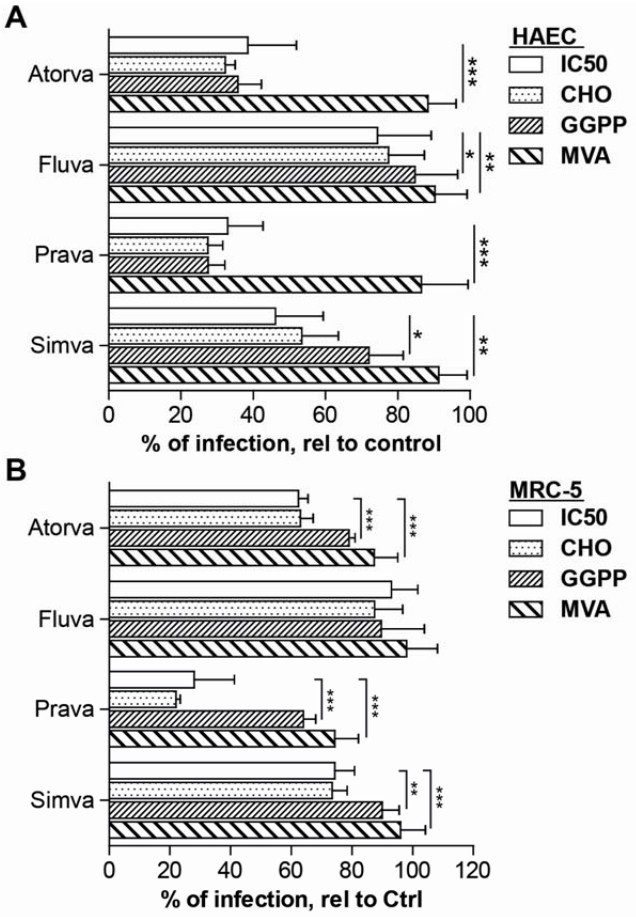

FIG 4. The anti-CMV activity of statins is mediated by mevalonate products.
Metabolite rescue experiments were realized in (A) HAEC and (B) MRC-5. HCMV infectious titers were determined at 6 dpi by a TCID50 assay in supernatant of cultures treated with the statins IC50 alone or co-treated with cholesterol (CHO) (1 µM), geranylgeranyl pyrophosphate (GGPP) (10 µM), or mevalonate (MVA) (100 µM). Results are expressed in relative level of infection compared to untreated cultures, and bars represent mean values ± SD of at least three independent experiments. Comparisons were performed using analysis of variance followed by Fisher's exact test and significant differences are indicated in the graph as follows: *$p < 0.05$,**$p < 0.01$,***$p < 0.001$.

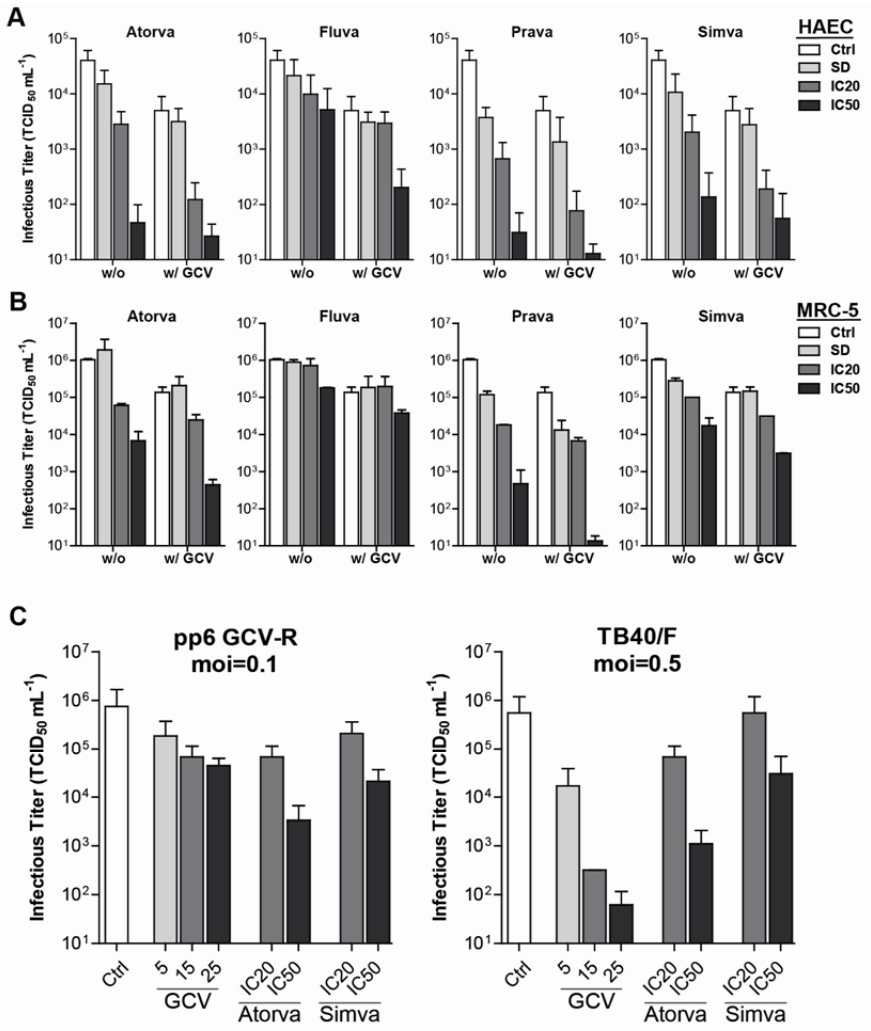

FIG 5. Statins enhance inhibition of HCMV replication by ganciclovir and inhibit the replication of GCV-resistant HCMV strain.

HCMV infectious titers were determined at 6 dpi by a TCID50 assay in supernatant of (A) HAEC and (B) MRC-5 cultures treated with the indicated doses of statin alone (w/o) or co-treated with ganciclovir (5 µM for HAEC, 1 µM for MRC-5) (w/ GCV). Results are expressed in TCID50 mL-1 as mean values ± SD of at least two independent experiments. (C) HCMV infectious titers were determined at 6 dpi by a TCID50 assay in supernatant of MRC-5 cultures treated with ganciclovir (5, 15 and 25 µM), atorva or simvastatin (IC20 or IC50) and infected with the pp6 GCV-resistant strain (MOI=0.1, left panel) or with the wild type TB40/F strain (MOI=0.5, right panel). Results are expressed in TCID50 mL-1 as mean values ± SD of two independent experiments.

Table 1. *In Vitro* anti-proliferative and anti-CMV activity of statins.

Cell type	Drug	Anti-proliferative activity[a] (μM)			Anti-CMV activity[b] (μM)	
		SD	IC_{20}	IC_{50}	EC_{50}	EC_{90}
HAEC	Atorvastatin	0.01	0.5	2	0.03 ± 0.05	0.17 ± 0.10
	Fluvastatin	0.01	0.1	0.3	0.03 ± 0.03	0.31 ± 0.18
	Pravastatin	50	100	400	26 ± 8	64 ± 20
	Simvastatin	0.01	0.5	1	0.003 ± 0.006	0.1 ± 0.08
MRC-5	Atorvastatin	0.1	1.5	5	0.46 ± 0.27	1.45 ± 0.57
	Fluvastatin	0.01	0.2	1	0.68 ± 0.37	4.08 ± 1.52
	Pravastatin	100	300	1000	55 ± 36	211 ± 116
	Simvastatin	0.1	1	3.5	0.16 ± 0.23	1.57 ± 1.04

[a] Concentrations responsible for 50% (IC50), 20% (IC20) or no cell growth inhibition (SD or subinhibitory dose) over a 72-hour period of time, as compared with untreated control cell growth.
[b] EC_{50} and EC_{90} values were determined by the indicated antiviral assay. Nonlinear regression analysis was performed, and the resulting graphs were used to calculate the respective values. Results are expressed as means ± standard deviations of at least 3 independent experiments.

Table 2. HCMV resistant strain to GCV remains susceptible to statins.

	pp6		TB40/F	
	EC_{50}	EC_{90}	EC_{50}	EC_{90}
Ganciclovir	7.3	140	1.2	2.9
Atorvastatin	0.80	1.6	0.96	1.5
Simvastatin	1.7	2.4	1.2	1.8

Data are in μM.

4. Curriculum Vitae

First name(s), surname	Nicolas PONROY
Date of birth	17.01.1985
Nationality	French

Education

10/2009 – 01/2014	**University of Zürich, *Switzerland*** **PhD student** Group of Prof. Dr. med. Nicolas J. Müller, Division of Infectious Disease and Hospital Epidemiology, University Hospital of Zürich Thesis title: "Statins Inhibit Cytomegalovirus Replication by Interfering with the Isoprenoid Arm of the Mevalonate Pathway"
2002-2007	**Ecole de Biologie Industrielle, *Cergy-Pontoise, France*** **Master of Science and Engineering Degree in Biology (12-2007)** Thesis title: "Preclinical study in an ovine model for tolerance and efficacy of Chondrocell®, an autologous cellular therapy product for cartilage repair"
1999-2002	**High School Richelieu, *Rueil-malmaison, France*** **Scientific Baccalauréat, Biology option (06-2002)**

Awards / Presentation / Publication

09-2013	"Statins Demonstrate a Broad Anti-Cytomegalovirus Activity in vitro in Ganciclovir-susceptible and resistant Strains" Ponroy N, Taveira A, Mueller NJ, Millard AL. *Manuscript submitted to the Journal of Medical Virology*
2012	Awarded by a research grant of the University of Zürich, 2012
2010	Speaker at the ""Antivirals Congress", November 7-9,2010, Amsterdam

Lectures

2013	Dealing with publication process, ETH Zürich
2012	Immunology I & II, ETH Zürich
2012	Study design & data analysis using statistical software R-1, University of Zürich

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